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Microbial consortia as inoculants for improved crop performance

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“Ich empfand das Leben nicht mehr als Selbstverständlichkeit; ich empfand es als ein seltenes Geschenk, das man auszunutzen verpflichtet ist.“

(Margarete von Wrangell)

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List of Abbreviations

ACC-deaminase	1-aminocyclopropane carboxylic acid deaminase
AHLs	N-acyl-homoserine lactones
AMF	arbuscular mycorrhiza fungi
BFDC	*Pennicilium sp.* PK 112 (surfactant dispersion)
BFOD	*Pennicilium sp.* PK 112 (oil dispersion)
BNF	biological nitrogen fixation
BE	bio-effector
BS	biostimulant
CAN	calcium-ammonium nitrate
CFA	Combifector A
CFB	Combifector B
cfu	colony forming unit
CHT	biopolymer chitosan
C-loess	loess material taken from the calcareous C-horizon (subsoil) of a luvisol
CULTAN	Controlled Long-Term Ammonium Nutrition
DAS	days after sowing
DCD	dicyandiamide
DM	dry matter
DMPP	3, 4-Dimethylpyrazole phosphate
DNA	deoxyribonucleic acid
DW	dry weight
EM	effective microorganisms
FAO	food and Agriculture Organization of the United Nations
Fig.	figure
HS	humic substances
HT	harvest time
IAA	indole-3-acetic acid

ICP-OES	inductively coupled plasma optical emission spectrometry
ISR	induced systemic resistance
MCP	microbial consortia product
PGPM	plant growth-promoting rhizobacteria
PH	protein hydrolysate
P_{org}	organic phosphate
qPCR	real time quantitative polymerase chain reaction
QS	quorum sensing
Red	reduced
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	rock phosphate
SD	standard deviation
SE	standard error
SEM	standard error of the mean
SOD	superoxide dismutase
sp.	species (singular)
SPAD	unites based on differences in optical density at two wavelengths as measured with a chlorophyll meter (Minolta SPAD-502)
spp.	species (plural)
Std	standard
T-22	*Trichoderma harzianum* T-22
Tab.	Table
var.	variety
VOCs	volatile organic compounds
w/w	weight per weight
WHC	water holding capacity

1 Summary

The use of microbial consortia products (MCP) based on combinations of different strains of plant growth-promoting microorganisms (PGPM) and frequently also on non-microbial bio-stimulants (BS) with complementary beneficial properties, is discussed as a strategy to increase the efficiency and the flexibility of BS-based crop production strategies under variable environmental conditions. Moreover, MCP application aims at the restoration of plant-beneficial, soil biological processes disturbed by soil degradation and intensive use of agro-chemicals. This PhD thesis was initiated to characterize the modes of action and the potential advantages of a representative commercial MCP formulation over selected single strain PGPM inoculants, with documented effects on plant growth promotion and pathogen suppression. In total, nine pot and field experiments were conducted with three crops (maize, spring wheat, tomato) on seven different soils with three organic and inorganic fertilization regimes.

MCP interactions with mineral fertilizers: A first set of pot experiments was conducted under controlled greenhouse conditions with maize as a model plant, to investigate MCP interactions with mineral N and P fertilizers. Nitrate fertilization was compared with the application of ammonium fertilizers, frequently used as N starter supply in maize cultivation systems. Nitrification inhibitors were employed to ensure a longer-lasting ammonium effect. The experiments were conducted on five soils with moderate to low P availability and a pH range between 5.9 and 7.9, with native soil P, soluble CaH_2PO_4 or sparingly soluble rock-phosphate (Rock-P) as P sources. Generally, beneficial MCP effects on plant growth were most strongly expressed in combination with stabilized ammonium fertilization, particularly under conditions of moderately low mineral P availability (20-30 mg kg^{-1} substrate), supplied as soluble fertilizer P or in form of native soil P (Bradáčová et al., 2019a, b). The ammonium effect was obviously related with increased P solubility due to ammonium-induced rhizosphere acidification. Phosphate solubilization was even detectable on a moderately acidic soil at pH 5.9 (Bradáčová et al., 2019b). By contrast, the additional MCP effect was rather associated with root growth promotion, which was not detectable in the ammonium treatments without MCP inoculation. However, the expression of beneficial MCP effects on root elongation was also dependent on the presence of ammonium and consequently on the efficiency of the nitrification inhibitor DMPP (Bradáčová et al., 2019b). Increased root length development in the MCP variants mediated improved spatial acquisition of P and also of other nutrients. By contrast, there was no indication for direct P solubilization from sparingly soluble Ca-phosphates induced by the MCP inoculant in the maize rhizosphere. Root growth was obviously stimulated by microbial

auxin supply provided by the MCP inoculant. This was indicated by increased auxin production of bacteria re-isolated from the rhizosphere of MCP-inoculated plants, particularly in combination with stabilized ammonium fertilization (Bradáčová et al, 2019a) and by increased expression of the AuxIAA5 gene in the root tissue, known to be rapidly activated by external auxin supply. By contrast, the expression of the PIN1c auxin transporter gene, rather activated by internal auxins during basipetal auxin transport, remained unaffected by MCP inoculation (Bradáčová et al., 2019b). Similar effects have been reported also in previous studies for various single strain inoculants and single strain combinations. based on fungal and bacterial genera including *Bacillus, Paenibacillus, Pseudomonas*, *Streptomyces, Trichoderma* and *Penicillium,* at least partially present also in the MCP formulation. However, a general comparison revealed no superior performance of the MCP inoculant in terms of plant growth promotion over the investigated single-strain inoculants. There was also no indication for MCP effects on marker enzyme activities involved in C, N and P cycling in the maize rhizosphere, related with plant growth promotion. This indicates a limited direct or indirect impact of the MCP inoculation on these processes, e.g. by interactions with the soil microbiome (Bradáčová et al., 2019a, b). By contrast, a follow-up study demonstrated improved P acquisition of tomato after MCP inoculation combined with stabilized ammonium fertilization, in a drip-irrigated field experiment conducted in the Negev desert in Israel. Under these conditions, significant microbiome effects were detectable even three months after the last MCP inoculation (Bradáčová et al., 2019c). An increased bacterial alpha-diversity at the rhizoplane was associated with a reduced abundance of *Sphingobacteriia,* known as salinity indicators and an increase in the population density of potentially plant-growth-promoting *Flavobacteriia*. However, also in this case it was not clear. whether these effects must be regarded as a cause or rather as a consequence of the improved P status of the host plants, induced by MCP inoculation (Bradáčová et al., 2019c).

MCP interactions with organic fertilizers: Improved utilization of N-rich organic fertilizers, such as composted manures and meat-meals, has been repeatedly demonstrated in combinations with various single strain inoculants described above. In this thesis, a similar study was initiated in Timisoara, Romania to compare the performance of selected single strain inoculants, and strain combinations of fungal and bacterial origin (*Penicillium* sp.; Proradix: *Pseudomonas sp*. DSMZ 13134; Rhizovital: *Bacillus velezensis* FZB42) with MCP treatments, over two years in tomato greenhouse production trials. Applied fertilizers were based on composted cow manure (nursery stage) and guano, hair-, and feather-meals during the production phase (Bradáčová et

al., 2019c). The BS treatments consistently increased tomato yields compared with the non-inoculated controls over two years. Beneficial effects were detectable already during early growth in the nursery phase, followed by stimulation of flowering and higher yield and improved fruit size distribution, even under conditions of increased pathogen pressure (*Fusarium oxysporum*, *Agriotes lineatus*) during the first year. The cumulative yield increase ranged between 39 and 84%, but without superior performance of the MCP or strain combinations over the single strain inoculants. Also in a follow-up study with spring wheat on a clay loam soil pH 5.9 with low P availability but high organic matter content, there was no indication for improved utilization of an organic fertilizer based on poultry manure and meat-meal by MCP inoculation, both, under field conditions and in a pot experiment (5.2). In the latter case, superior performance was recorded even for a single *Bacillus simplex* CH13 strain. However, in these experiments, water limitation was included as additional stress factor.

MCP performance under stress conditions: In the experiments conducted in this thesis, plants and inoculants were intentionally or unintentionally exposed to a range of stress factors, including drought, (5.2; Neundorf, 2018), high temperatures and severe P limitation (Bradáčová et al., 2019c), potential toxicities due to high manure contents of nursery substrates and soil acidity (Bradáčová et al., 2019bc) and increased pathogen pressure (Bradáčová et al., 2019c). Beneficial effects of MCP inoculation on plant growth and yield formation were detected in five experiments, exclusively under conditions when plant cultivation was performed completely or at least partially under protected greenhouse conditions, particularly during the sensitive rhizosphere establishment phase of the inoculants. In most cases without MCP effects, the plants were exposed to stress factors affecting root development such as extreme P deficiency during early growth, acidic rhizosphere pH, Ca limitation, and drought stress (5.2; Neundorf, 2018; Bradáčová 2019b). Under these conditions even multiple inoculant strains with differences in stress tolerance will have only a limited advantage, as long as the stress conditions affect the ability of the host plant to support the establishment of a functional MCP interaction in the rhizosphere. Since this scenario is more likely in agricultural crops directly sown under field conditions as compared with greenhouse or nursery cultures, it remains a major challenge for practical applications.

Only in one out of nine experiments conducted in this thesis, clear evidence for superior MCP performance was detectable in a drip-irrigated tomato field experiment conducted under the challenging environmental conditions of the Negev desert in Israel (Bradáčová et al., 2019c). This finding demonstrates that MCP inoculants can exhibit an advantage over single strain

inoculants but not as a general feature. Selective interactions with the type and dosage of the selected fertilizers, as well as avoidance of inhibitory effects on root growth during MCP rhizosphere establishment, have been identified as critical factors. A further characterization of the conditions, promoting beneficial plant-MCP interactions is mandatory for a more targeted and reproducible MCP application.

2 Zusammenfassung

Die Nutzung mikrobieller Konsortien (MCP) auf Basis unterschiedlicher Stämme pflanzenwachstums-stimulierender Mikroorganismen (PGPMs), oft auch in Verbindung mit nichtmikrobiellen Biostimulanzien (BS), mit komplementären, nützlichen Eigenschaften wird als Ansatz diskutiert, die Effizienz BS-unterstützter Produktionssysteme im Nutzpflanzenanbau unter variablen Umweltbedingungen zu verbessern. Darüber hinaus soll die Anwendung von MCPs zur Regeneration gestörter bodenbiologischer Prozesse beitragen, die durch Bodendegradation und intensive Nutzung von Agrochemikalien hervorgerufen werden können. Die vorliegende Arbeit hatte das Ziel die Wirkmechanismen und die potenziellen Vorteile einer repräsentativen, kommerziellen MCP Formulierung, gegenüber Einzelstamm-Inokulanzien mit nachgewiesener pflanzenwachstums-stimulierender und pathogen-suppressiver Wirkung zu charakterisieren. Insgesamt wurden 9 Topf-, und Feldversuche mit 3 Kulturpflanzenarten (Mais, Sommerweizen, Tomate) auf 7 unterschiedlichen Böden und 3 organischen und mineralischen Düngungsregimes durchgeführt.

MCP-Interaktionen mit Mineraldüngern: Zur Untersuchung von MCP-Interaktionen mit mineralischen N-. und P-Düngern, wurden Topfversuche mit Mais als Modellpflanze durchgeführt. Reine Nitratapplikation wurde mit Ammonium-dominierter Düngung, wie sie verbreitet als Starterdüngung im Maisanbau eingesetzt wird, verglichen. Nitrifikationsinhibitoren wurden zur Ammoniumstabilisierung eingesetzt. Die Versuche wurden auf 5 Böden mit moderater bis niedriger P-Verfügbarkeit, einem pH Bereich zwischen pH 5,9 -7,9 und nativem Bodenphosphat, sowie löslichem CaH_2PO_4 bzw. schwerlöslichem Rohphosphat als P-Quellen, durchgeführt.

Generell waren fördernde MCP-Effekte auf das Pflanzenwachstum besonders stark in Kombination mit stabilisierter Ammoniumdüngung unter Bedingungen mit niedriger bis moderater P-Verfügbarkeit (20-30 mg kg^{-1} Substrat) ausgeprägt (Bradáčová et al., 2019a, b). Der Ammoniumeffekt stand offensichtlich im Zusammenhang mit einer P-Mobilisierung durch Ammonium-induzierte Ansäuerung der Rhizosphäre, die selbst auf leicht sauren Böden mit pH 5,9 noch nachweisbar war (Bradáčová et al., 2019b). Im Gegensatz dazu, war ein zusätzlicher MCP Effekt eher durch Stimulierung des Wurzelwachstums bedingt, was ohne MCP Inokulation nicht nachweisbar war. Andererseits war die MCP-induzierte Wurzelwachstumsförderung aber auch abhängig von der Gegenwart von Ammonium und damit von der Wirksamkeit des eingesetzten Nitrifikationshemmstoffes DMPP (Bradáčová et al.,

2019b). Das verbesserte Wurzelwachstum unterstützte die räumliche Aneignung von Phosphat aber auch von anderen Nährstoffen, allerdings gab es keine Hinweise auf eine direkte MCP Wirkung durch Mobilisierung schwerlöslicher Ca-Phosphate (Bradáčová et al., 2019b). Das Wurzelwachstum wurde offensichtlich durch mikrobielle Auxinproduktion gefördert. Entsprechend zeigten Bakterienpopulationen, die nach MCP-Inokulation aus der Maisrhizosphäre isoliert wurden, erhöhte Auxinproduktion besonders in Kombination mit stabilisierter Ammoniumdüngung (Bradáčová et al, 2019a), und die Expression des AuxIAA5 Gens, die besonders durch externe Auxinapplikation gefördert wird, war nach MCP-Inokulation im Maiswurzelgewebe erhöht. Dagegen wurde die Expression des PIN1c Auxintransportergens, das beim basibetalen Auxintransport eher durch endogenes Auxin aktiviert wird, durch MCP-Inokulation nicht beeinflußt (Bradáčová et al., 2019b). Ähnliche Effekte wurden in früheren Studien auch mit pilzlichen und bakteriellen Einzelstamm-Inokulantien und Stamm-Kombinationen auf Basis der Gattungen *Bacillus, Paenibacillus, Pseudomonas, Streptomyces, Trichoderma* und *Penicillium* berichtet, die zum Teil auch Bestandteile der MCP Formulierung bilden. Ein Übersichtsvergleich ergab dabei allerdings keine verbesserte MCP-Wirkung im Vergleich zu den Einzelstammiokulanzien (6.1). Es gab auch keine Hinweise auf MCP Effekte in Bezug auf die Aktivität vom Markerenzymaktivitäten für die Umsetzung von C, N und P in der Rhizosphäre im Zusammenhang mit der pflanzenwachstums-fördernden Wirkung. Daher kann nicht von einer signifikanten direkten oder indirektem MCP Wirkung auf die betreffenden Prozesse z.B. über Interaktionen mit dem Bodenmikrobiom ausgegangen werden (Bradáčová et al., 2019a, b). Allerdings zeigte auch ein Folgeexperiment zum Feldanbau von Tomate mit Tröpfchenbewässerung, in Kombination mit stabilisierter Ammoniumsaulfatdüngung in der Negev-Wüste in Israel, verbesserte P Aneignung und Ertragsbildung nach MCP Inokulation. Unter diesen Bedingungen waren signifikante Mikrobiomeffekte auch noch drei Monate nach der MCP Inokulation nachweisbar (Bradáčová et al., 2019c). Die MCP Varianten zeigten eine erhöhte Alphadiversität bakterieller Populationen an der Wurzeloberfläche, verbunden mit einer verminderten Abundanz von *Sphingobacteriia*, die z.B als Salzstressindikatoren bekannt sind und einer erhöhten Abundanz potenziell pflanzenwachstumsfördernder *Flavobacteriia*. Allerdings ist in diesem Fall nicht klar, ob die beobachteten Mikrobiomeffekte die Ursache oder eher eine Folge des verbesserten P Ernährungsstatus der Tomatenpflanzen nach MCP Inokulation repräsentieren (Bradáčová et al., 2019c).

MCP-Interaktionen mit organischen Düngern: Eine verbesserte Nutzung N-reicher organischer Dünger z.B. auf Basis Stallmistkompost oder Fleischmehlen durch die oben beschriebenen Einzelstamminokulanzien wurde verschiedenen Vorgängerstudien belegt. Daher wurde in der vorliegenden Arbeit eine Studie mit verschieden bakteriellen und pilzlichen Einzelstamm-Inokulanzien und Stammkombinationen (*Penicillium sp.*; Proradix: *Pseudomonas sp.* DSMZ 13134; Rhizovital: *Bacillus velezensis* FZB42) im Vergleich zu MCP Behandlungen in der Gewächshaustomatenproduktion in Timisoara, Rumänien durchgeführt. Die verwendeten organischen Dünger umfassten Rindermistkompost in der Anzuchtphase und Guano, Haar-, und Federmehle in der Hauptkultur (Bradáčová et al., 2019c). Die Inokulation führte zu konsistenten Ertragssteigerungen über zwei Jahre im Vergleich zur unbehandelten Kontrolle. Fördernde Effekte auf das vegetative Wachstum waren bereits in der Anzuchtphase nachweisbar, gefolgt von stimulierter Blütenbildung, Ertragserhöhung und verbesserter Fruchtgrößenverteilung, sogar unter einem erhöhten Krankheitsdruck (*Fusarium oxysporum*, *Agriotes lineatus*) während des ersten Versuchsjahres. Die kumulative Ertragssteigerung lag zwischen 39 und 84%, wobei die MCP Behandlungen keine verbesserte Wirkung gegenüber den Einzelstämmen oder den Stammkombinationen zeigten. Auch in einem Folgeexperiment mit Sommerweizen auf einem tonigen Lehmboden pH 5,9 mit hohen C_{org} Gehalt, geringer P Verfügbarkeit und organischer Düngung auf Basis von Geflügelmist und Fleischmehl, ergaben sich keine Hinweise auf eine verbesserte MCP Wirkung sowohl im Feldversuch, als auch im Topfexperiment (5.2). Im letzteren Fall zeigte sogar ein Einzelstammpräparat auf Basis von *Bacillus simplex* CH13 die beste Wachstumswirkung. Allerdings war in diesen Versuchen Wassermangel ein zusätzlicher Stressfaktor.

MCP-Wirkungen unter Stressbedingungen: Im Rahmen der Versuche der vorliegenden Studie kamen beabsichtigt oder unbeabsichtigt auch verschiedene Stressfaktoren zum Tragen, wie z.B. Wassermangel (5.2; Neundorf, 2018), hohe Temperaturen und starker P-Mangel (Bradáčová et al., 2019c), mögliche Substrattoxizität durch hohe Stallmistgehalte und niedrige pH-Werte (Bradáčová et al., 2019b, c), sowie erhöhter Pathogendruck (Bradáčová et al., 2019c). Pflanzenwachstumsfördernde und ertragssteigernde MCP Wirkungen wurden in 5 Versuchen erzielt, und zwar nur dann, wenn zumindest die Vorkultur der Pflanzen während der empfindlichen MCP-Etablierungsphase in der Rhizosphäre, unter geschützten Gewächshausbedingungen durchgeführt wurde. In den meisten Fällen ohne MCP-Wirkung waren die Pflanzen Stressfaktoren mit hemmender Wirkung auf das Wurzelwachstum, wie extremem P Mangel während der Keimlingsentwicklung, niedrigem Boden pH und Ca-Mangel

oder Trockenstress ausgesetzt (5.2; Neundorf, 2018; Bradáčová 2019b). Unter diesen Bedingungen bringen auch MCP Formulierungen mit unterschiedlich stresstoleranten PGPM Stämmen keinen entscheidenden Vorteil, sofern die vorherrschenden Stressfaktoren die Fähigkeit der Wirtspflanze beschränken, die MCP-Etablierung in der Rhizosphäre zu unterstützen. Dieses Szenario ist wahrscheinlicher bei Ackerbaukulturen mit direkter Aussaat im Freiland im Vergleich zu Gewächshaus-, oder Vorkulturanzucht und stellt so eine entscheidende Herausforderung für die praktische Anwendung dar.

Nur in einem von neun Versuchen der vorliegenden Studie gab es eindeutige Hinweise auf eine verstärkte Ausprägung von Wachstums-, und Ertragseffekten durch MCP Inokulation beim Feldanbau von Tomaten mit Tröpfchenbewässerung unter den verhältnismäßig ungünstigen Umweltbedingungen in der Negev-Wüste in Israel (Bradáčová et al., 2019c). Diese Beobachtung zeigt, dass eine generell verbesserte Wirksamkeit von MCP Formulierungen gegenüber Einzelstamm-Inokulanzien nicht gegeben ist. Selektive Interaktionen mit der Art und der Menge der eingesetzten Düngemittel und die Vermeidung von Stresswirkungen mit hemmendem Einfluss auf die Wurzelentwicklung während der Etablierungsphase wurden als kritische Faktoren identifiziert. Eine umfassendere Charakterisierung der Bedingungen, die eine erfolgreiche MCP-Interaktion mit der Wirtspflanze begünstigen ist daher unumgänglich für zielgerichtete und reproduzierbare MCP-Anwendungen in der Praxis.

3 General introduction

3.1 The need for alternative sustainable agriculture

In natural ecosystems, soil fertility and healthy plant growth essentially depend on mutual interactions with beneficial soil macro- and microbiota, supporting plant nutrition and resilience to biotic and abiotic stresses. The ability of host plants to recruit specific communities of beneficial soil biota via root activities is a key factor for the exploitation of ecological niches differing in soil properties and forms of nutrient supply. Consequently, these interactions provide a major driving force, determining soil fertility, plant performance as well as above- and below-ground biodiversity (Roy et al., 2006; Glick 2014).

Conventional agriculture, focused on maximizing economic outputs with a narrow range of crop varieties, systematically replaces the beneficial biodiversity interactions by external inputs (i.e. agro-chemicals, tillage). Consequently, beneficial ecosystem functions of soil biota governing soil fertility by their multi-faceted roles in nutrient cycling, soil structure building, and stress resilience, are declining. This is associated with ecological risks, such as eutrophication, greenhouse gas emissions, and excessive consumption of non-renewable natural resources. Increasing erosion, soil salinization, drought, compaction and chemical pollution and can further aggravate this scenario. Growing awareness of these negative side effects raises societal and political interest in the development of alternative, more sustainable and eco-efficient production strategies (Rengel and Marschner 2005; Swaminathan 2006; Glick 2014). However, recovering mutualistic plant-soil biota interactions can take many years (e.g. build-up of organic matter, soil structure, mutualistic soil life, pathogen-antagonist equilibria). Moreover, alternative approaches, such as organic farming, or other concepts of regenerative agriculture frequently trade-off against lower yields due to the more limited flexibility to adapt fertilizer supply and plant protection to actual crop demands. Attempts to close these gaps can further increase the land use intensity for agricultural production, thereby reducing potential ecological benefits (Muller et al., 2017). Against this background, there is an urgent need to develop bio-ecological strategies linking the benefits of soil bio-diversity with sustainable agricultural soil/crop management for an ecological intensification of agriculture. To achieve these ambitious goals, not only the augmentation of autochthonous soil life by an adapted crop/soil management needs to be taken into consideration (Roy et al., 2006; Glick 2014). Particularly for the regeneration of already affected soils, the introduction of appropriate bioeffectors or biostimulants (BS: i.e. plant-beneficial microorganisms and active natural

compounds) in strategic combination with compatible fertilizers promoting the establishment and expression of beneficial traits may provide an additional approach, to promote the re-establishment of beneficial belowground ecosystem services into agricultural production systems (Rengel and Marschner 2005; Richardson 2009; Bashan et al. 2014; Glick 2014). However, stress factors affecting plant and root development (e.g. excessive use of agrochemicals, pathogens, adverse climate and soil factors) and genotypic variation in rhizosphere competence of introduced BS can complicate the effective expression of beneficial BS traits, leading to variable results. Currently this scenario still represents a major challenge for BS-assisted production strategies (Neumann et al., 2009, Bashan et al. 2014).

3.2 PGPM in general and their modes of action

So-called biostimulants (BS) are a group of living plant-growth promoting bacteria, fungi (such as arbuscular mycorrhiza fungi) and active natural substances of inorganic (silicates, chitosan) and organic (algae extracts, humic and fulvic acids) origin, which can demonstrate direct or indirect positive effects on plant performance. Biostimulants do interact with plants in various rhizosphere biological and biochemical processes and are involved in complex soil-plant-microbe interactions. The biostimulants are not supposed to directly bring relevant amounts of nutrients into the system, neither in their organic or inorganic form. The bacterial strains adopting direct bio-control and plant disease-eliminating effects for instance due to antibiotics production, are strictly excluded from the definition of biostimulants (Jardin, 2015). However, the biostimulants plus the strains possessing indirect plant disease-reducing effects are termed as so called “bio-effectors” (BEs) (BIOFECTOR Periodical Report 2012).

Generally, biostimulants adopt different functional mechanisms and different modes of action with resoect to their ability for plant-growth promotion. For instance, the stimulation of root and shoot plant growth and better plant establishment could be in some cases attributed to an increased microbial phytohormone production, to the stimulation of phytohormone production by the plant or to the production of various enzymes involved in C, N and P turnover in the rhizosphere, or to the production of secondary metabolites by the plant itself. Moreover, the biostimulants can induce an improvement of the plant nutritional status without having a direct fertilization effect for example by microbial N_2-fixation; phosphorus mineralization and mobilization of sparingly available nutrients, for instance by the release of siderophores and carbocylates. Additionally, the biostimulants adopt certain biocontrol strategies such as enhancement of the plant vitality and health, tolerance to biotic stresses by the induction of systemic plant-resistance, production of antibiotic substances, or competition for space.

Specifically, biostimulants based on organic natural substances, such as algae extracts (mainly based on *Ascophyllum nodosum*) containing different phytohormones and amino acids, having the potential to increase plant growth via improved root growth followed by increased nutrient solubility especially under abiotic stress conditions (Halpern et al., 2015; Van Oosten 2017). The main biofertilizing, biostimulating and biocontrol properties of biostimulants are summarized in **Fig. 1**.

3.2.1 The rhizosphere and plant-microbe interactions

The establishment of beneficial plant-microbe interactions plays a key role in nutrient acquisition and stress resistance of higher plants. A large number of soil microorganisms is commonly found in the rhizosphere directly attached to the plant roots but also at the root surface (rhizoplane) and even inside root and shoot tissues (endophytes). The rhizosphere, representing the soil volume affected by root activity as defined by Lorenz Hiltner in 1904 (Hartmann, 2008), is usually enriched in organic compounds as compared to the bulk soil. The rhizosphere accumulation of different sugars, organic acids, amino acids and peptides, enzyme proteins, exopolysaccharides, vitamins, phenolics and other secondary plant metabolites originating from rhizodeposition and root exudates, serves as carbon and nitrogen source for the microorganisms and enables them to proliferate and to be metabolically active. Therefore, the rhizosphere represents a very attractive but also selective hot spot for a high diversity of soil microorganisms, such as bacteria, algae, fungi and also microbial grazers, such as protozoa or nematodes, depending on the individual composition of the rhizodeposition in different plant species and cultivars. (Marschner 2012; Gopalakrishnan et al., 2015). The diverse group of rhizosphere-colonizing microorganisms, having a positive impact on plant growth is termed as plant growth-promoting microorganisms (PGPM), (Kloepper et al., 1991). Plant growth-promoting microorganisms are settled in the rhizosphere, are attached to the root or they may occupy the interior spaces inside of the host plants as so called endo-colonizers or endophytes (Kloepper at al., 1991; Glick 2014). Important groups of endophytes are represented by the intracellular bacteria forming and occupying root nodules, which have the ability to fix atmospheric nitrogen, such as the *Rhizobia* or Frankia group. Glomeromycota, Trichoderma and Sebacinales species as well as ecto- and endo- mycorrhiza fungi are examples of fungal PGPM endophytes. The main groups of different bacteria being considered as PGPMs comprise the phyla of Bacteroidetes, Firmicutes, Actinobacteria, Cyanobacteria and Proteobacteria. The generally known soil bacteria having plant growth-promoting properties are represented by the genera *Bacillus*, *Rhizobium*, *Bradyrhizobium*, *Pseudomonas*, *Azotobacter*, *Azospirillum*,

Acetobacter, *Burkholderia*, *Serratia*, *Enterobacter* including species such as *Bacillus velezensis (*former *amyloliquefaciens)*, *Bacillus simplex*, *Bacillus subtilis*, *Bacillus pumilus*, *Pseudomons fluorescens*, *Pseudomonas putida*, *Azospirillum brasilense*, *Paenibacillus polymyxa* and *Azotobacter vinelandii* and their specific strains (Glick 1995; Gopalakrishnan et al., 2015).

However, the expression of PGPM properties are frequently strain-specific traits.

3.2.2 General modes of action of PGPM

The promotion plant growth by PGPMs can be attributed to direct or more indirect effects. Direct growth promotion can be mediated improved nutrient acquisition, such as associative nitrogen (N) fixation of so called “diazotrophs” as well as liberation of phosphate (P) from either organically or inorganic soil P forms by so called “P-solubilizing microorganisms (PSMs)”. The P solubilisation may be attributed to the efflux of protons and organic anions such as gluconates, oxalate, malate or citrate and even mineral acids, or to the release of enzymes such as phosphatases and phytases (Richardson and Simpson, 2011; Calvo et al., 2014). In the bulk soil, the microbes are able to mineralize organic P and contribute thus very strongly to an increased plant-available P pool in the soil (Richardson et al., 2009). However, the activity of phosphatases is higher in the rhizosphere as compared to the bulk soil which refers to the higher microbial activity (rhizosphere effect) but also to the ability of plant roots to secrete acid phosphatases to mobilize P from organic sources in case of P deficiency. PGPM also adopt mechanisms of Fe mobilization by production of siderophores, which can increase the availability of Fe for root-induced Fe acquisition (Neumann and Römheld, 2007). Apart from nutrient mobilization, PGPMs can also directly stimulate plant growth by interactions with phytohormonal balances and signalling. This involves the production of volatile organic compounds (VOCs) with signal functions, certain quorum sensing signals and phytohormones such as Indole Acetic Acid (IAA), cytokinis, giberillins and/ or reduction of excessive stress-induced ethylene accumulation with growth inhibitory effects on plants by ACC-deaminase production. The direct root growth promotion (enhanced total root length, root branching, promotion of lateral roots and root hairs, etc.) may be induced by the production of phytohormones such as IAA (Yang et al., 2008; Saharand and Nehra, 2011; Richardson and Simpson, 2011). However, PGPM do not always produce phytohormones themselves, in some cases they may also influence the phytohormonal synthesis and signalling of the host plant (Lugtenberg and Kamilova, 2009; Richardson and Simpson, 2011).

Indirect plant growth promotion by PGPMs can be mediated by an improved tolerance to biotic and abiotic stresses which can be among other factors attributed to an improved plant nutritional status and improved soil water relationships by root growth stimulation. Also, stimulation of physiological plant defense responses against abiotic and biotic stress, including detoxification of reactive oxygen species, production of antioxidants and phytoalexins frequently with systemic effects (stress priming) are important mechanisms of indirect plant growth promotion. Furthermore, PGPM abilities such as the antibiotic production and suppression of well-known pathogens such as *Fusarium*, *Pythium* or *Rhizoctonia* (inducing severe plant diseases) or the competition against deleterious bacteria (inducing restricted plant growth) may contribute to better plant tolerance against biotic stresses and improved plant performance (Whipps 2001; Lucy et al., 2004; Yang et al., 2008). However, indirect PGPM effects are not only restricted to plant pathogen interactions, also stimulatory effects on the establishment of symbioses with other beneficial soil biota are documented e.g. for helper functions on mycorrhizal fungi (Yusran et al., 2009). The interactions between different PGPMs with the host plants and their major modes of action are summarized in **Fig. 1**. The list describing the most common PGPMs and their modes of action follows in **Tab. 1**.

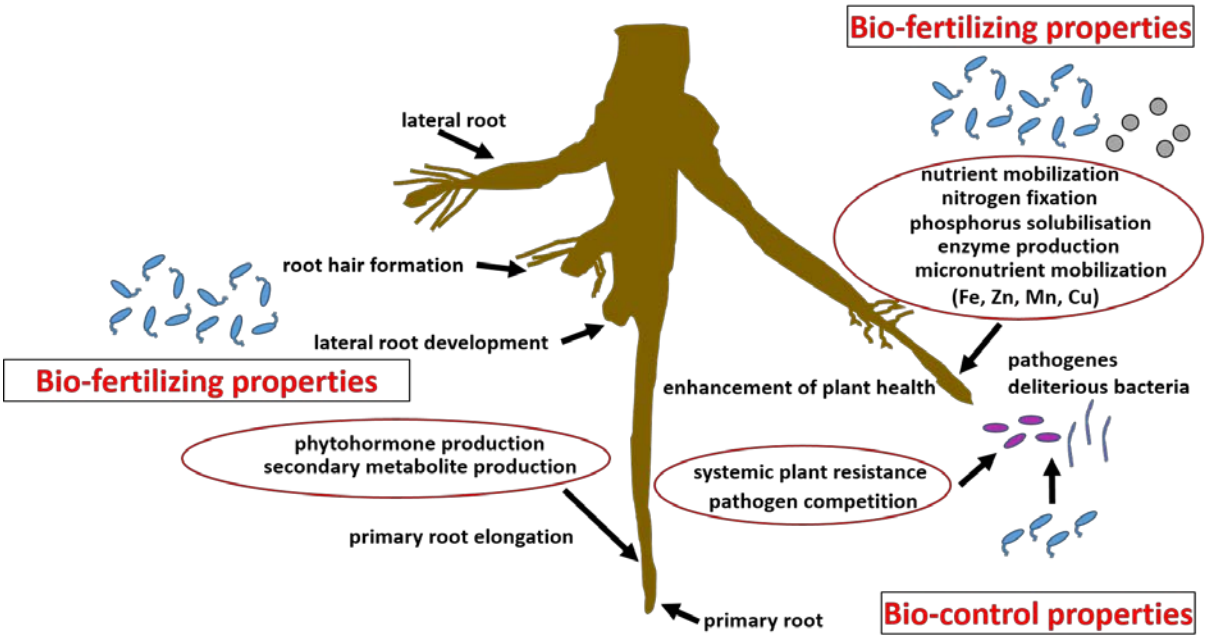

Fig. 1: Specific biofertilizing and biocontrol properties of biostimulants (modified after Vacheron et al., 2013).

Tab. 1: Major PGPMs with their suggested modes of action and proposed mechanisms for plant growth promotion.

Species	**Plant growth promoting properties and mechanisms**
Azospirillum spp.	- N_2-fixation - Production of phytohormone-like substances (Bloemberg & Lugtenberg, 2001; Halpern et al., 2015)
Azotobacter spp.	- N_2-fixation - Phosphate solubilisation - Production of phytohormone-like substances (Halpern et al., 2015)
Bacillus spp.	- Phosphate solubilisation - Production of phytohormone-like substances - Production of antibiotic substances - Successful colonization of plants - Beneficial effects on mycorrhizal symbioses (Bloemberg & Lugtenberg, 2001; Ramirez and Kloepper 2010; Bhattacharyya and Jha, 2012; Halpern et al., 2015)
Penicillium spp.	- Phosphate solubilisation - Production of antibiotics - Induction of systemic resistance in plants (ISR) - Successful root colonization - Cold-stress tolerance (Hossain et al., 2007; Gómez-Muñoz et al. (2018)
Pseudomonas spp.	- Phosphate solubilisation - Micronutrient mobilization due to release of siderophores - Production of phytohormones and contribution to phytohormonal balance - Production of antibiotics and anti-fungal metabolites - Induction of systemic resistance in plants (ISR) - Successful root colonization (competition for space) - Beneficial effects on mycorrhizal symbioses - (Glick et al., 1995; Bloemberg & Lugtenberg, 2001; Calvo et al., 2014; Halpern et al., 2015)
Rhizobium spp.	- Symbiotic N_2-fixation - Phosphate solubilisation - Micronutrients solubilisation due to production of siderophores - Beneficial effects on mycorrhizal symbioses (Bhattacharyya and Jha, 2012; Halpern et al., 2015)
Trichoderma spp.	- Phosphate solubilisation - Ensuring phytohormonal balances - Micronutrients solubilisation due to production of siderophores - Production of anbitiotic substances

- Inhibition of pathogens (mycoparasitism) - Induction of ISR and localized resistance - Beneficial effects on mycorrhizal symbioses - Increase of the N-fertilizer use efficiency - Improved drought stress tolerance (Harman, 2006; Calvo et al., 2014; Halpern et al., 2015)

3.2.3 Non-microbial biostimulants

3.2.3.1 Humic and fulvic substances

As reported in the literature, humic (HS) and fulvic (FS) substances used as biostimulants are of a relevant importance in agricultural and horticultural context. Humic and fulvic substances can have both direct and indirect positive effects on plant growth and improvement of plant performance. The direct effect on plant growth and plant development in response to HS is attributed to improved nutrient uptake. This is probably induced by the positive changes of root architecture or induction of lateral growth promotion which enables the plants to reach for soil nutrients from more distant sources. Additionally, HS directly interact with plant membrane transporters which are responsible for nutrient uptake and induce thus consequently improved growth and development of plants. Thereby, the nutrient content of HS itself is neglectable (Canellas et al., 2015). Furthermore, HS are involved in changes of primary and secondary metabolism of plants. For instance, plant growth can be promoted by the activation of C and N metabolism induced by HS. (Canellas et al., 2013; Hernandez et al., 2015). It was also shown that there was a higher accumulation of secondary metabolites such as phenolics in plants treated with HS. In this context, it is suggested that HS with their positive effects on secondary metabolites may also play a role in reduction of biotic and abiotic stresses. It was shown, that plants treated with humates are less susceptible to pathogens and have higher capacity to reduce drought and salinity stress. This may be mainly induced by the antioxidant defense mechanisms increased in the presence of HS, such as the stimulation of catalase and other enzymes resulting in reduction of peroxidation and scavenging of reactive oxygen species (ROS). Thus the plants become more tolerant against abiotic stresses, such as drought or salinity stress. HS are also able to improve plant growth and stress tolerance indirectly via the improvement of soil physical, chemical and biological properties (Paksoy et al., 2010; Hernandez et al., 2015; Canellas et al., 2015).

3.2.3.2 Plant and seaweed extracts

Usage of seaweed extracts having plant-growth promoting properties, could induce higher yields, increased uptake of nutrients and improved seed germination. Growth stimulation and increased uptake of minerals resulting in overall improved plant fitness also under unfavourable are the main advantages of such BS (Sharma et al, 2013; Omar et al., 2015). BS based on plant and seaweed extracts containing mainly the extracts of the algae *Ascophyllum nodosum* are known to improve especially plant tolerance against biotic and abiotic stresses. Similarly, as in the case of HS also the seaweed extracts are able to activate the antioxidant defense mechanisms and mitigate thus the oxidative stress on plants. Further, seaweed extracts are involved in specific root-microbe interactions and are able to improve root growth and nutrient uptake of plants as well as soil health. This can result in improved plant performance, enhanced plant growth and improved yield and fruit quality of crops (Shukla et a., 2019).

3.2.3.3 Chitosan polymers, amino acids and peptides

Chitosan polymers used as BS are mainly based on the biopolymer chitosan (CHT) coming mainly from the deacetylation of chitin. CHT seems to be an efficient BS with multiple advantages. Its production is relatively inexpensive and it can be easily combined with other substances to induce even better results on plant performance. Promotion of shoot growth, overall improved plant performance as well as higher fruit yields, fruit diameter or higher phenolic contents in fruits attributed to CHT application were observed in the literature. Apart from the direct positive effects on plant performance, nutrient uptake and improved yields, CHT also has the ability to reduce plant pathogens such as *Fusarium solani* or *Rhizoctonia solani.* Therefore, the usage of CHT as BS seems to be a successful strategy (Malerba and Cerana, 2018).

The mixture of different amino acids and peptides acting as BS belong to the group of BS called protein hydrolysates (PH). This group of BS receive an increasing attention during the last years thanks to its positive effects on plant performance. PHs are mainly produced by the hydrolysis of animal- or plant-derived protein materials. Increased nutrient uptake by plants as well as better transport of amino acids and peptides into the plants was observed after inoculation with PH. Positive effects on primary and secondary plant metabolism and increased tolerance to abiotic stresses was detected as well. PH are able to stimulate N metabolism and its assimilation and control thus plant growth and its development. Further, improved total yield and increased fruit size and number as well as a positive effect on nutrient uptake, mainly of

cationic nutrients such as K, Ca and Mg in the soil leading to the improved growth is attributed to the application of amino acids and peptides. Still, there is a lack of knowledge on the impact of PH on the soil microbial communities. The standardization of the PH-based products remains challenging as well. Therefore, a further research in this field is indeed necessary (Colla et al., 2015).

3.3 Characteristics of single component BS products, microbial combi-products and microbial consortia

Biostimulants can be divided into three main groups, depending on the complexity of their composition:

Single component: BS products or formulations composed of one specific bacterial, fungal, or non-microbial active ingredient with well-described characteristics and properties, with specific beneficial effects on plant performance. Targeted application of pre-selected strains is here a common practice.

Microbial combi-products: products or formulations with strictly defined content, composed of two or more bacteria, fungi or non-microbial biostimulant agents as well as the addition of stress protective nutrients (e.g. Zn, Mn, Cu, B, Si etc.). Additional positive effects on plant growth induced by synergistic interactions between the specific components are expected.

Microbial consortia: products or formulations based on many different, not strictly defined species of bacteria, fungi, algae extracts, amino and humic acids, chitin residues and many different organic substances frequently produced by fermentation or composting of various organic materials and waste products. During the fermentation different undefined species and strains can be proliferated and are in some cases supplemented also with selected single strain inoculants and non-microbial biostimulants or stress protective nutrients. An increased soil microbial diversity and broader field of usage of these products due to many different beneficial properties coming from different compounds of the consortia is expected.

The different groups of biostimulants according to their composition and specific modes of action are demonstrated in **Fig. 2**.

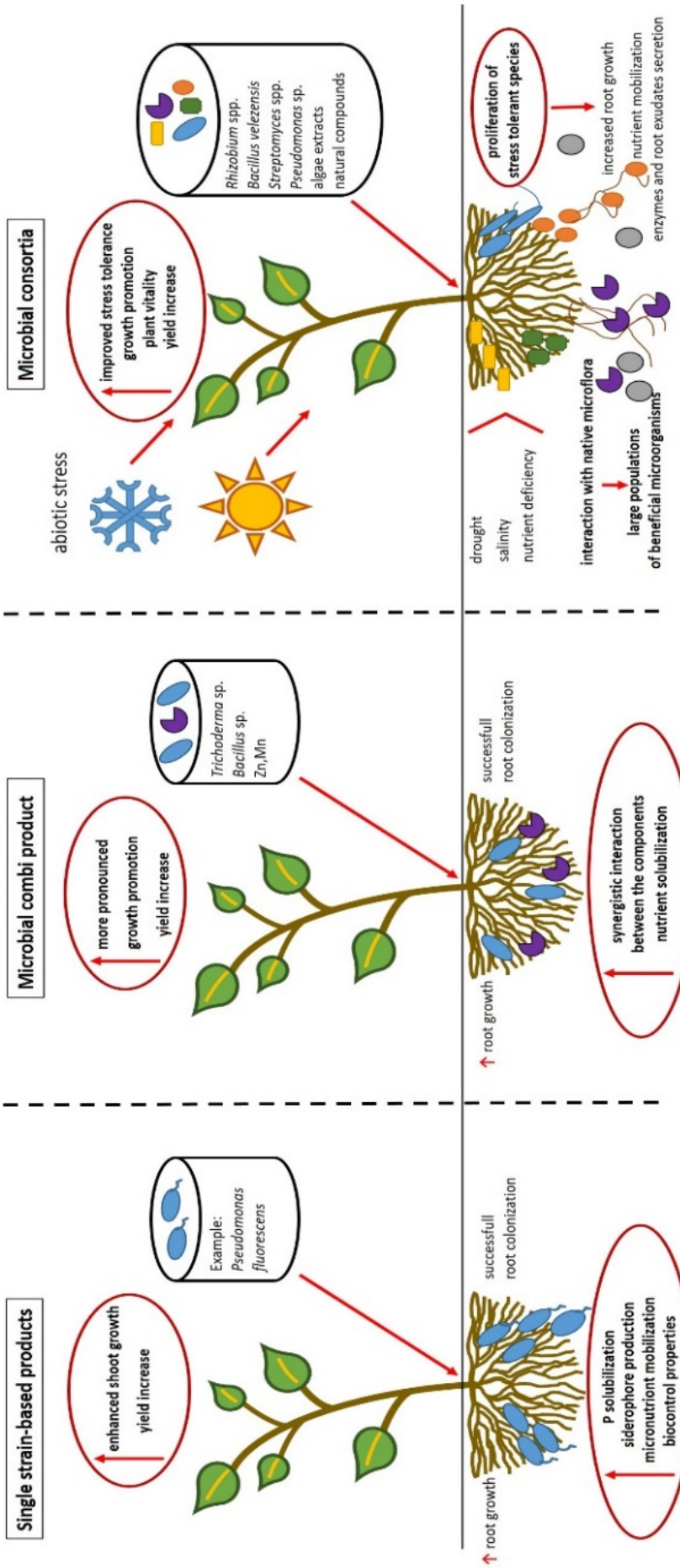

Fig. 2: Different possible scenarios of plant growth promotion depending on the type of the biostimulants.

3.3.1 Examples for important single component biostimulants

During the BIOFECTOR EU Project, different biostimulants based on single PGPM strains have been chosen in order to test their effects on plant growth. The general application strategy of such biostimulants is usually based on the selection of specific microbial strains with high efficiency of plant growth promotion under specific conditions such as limited P availability, restricted root growth, pathogen pressure etc. (http://www.biofector.info, 10.8. 2018). These microbial inoculants consist always of one specific, pre-selected microbial strain with its known and expected plant-growth promoting properties. The bacteria and fungi are cultivated separately, then removed from the fermentation process, concentrated and formulated into the form of final product (Calvo et al., 2014). The promising microbial biostimulants belonging to this category various bacterial and fungal strains such as: *Penicillium bilaii (P. bilaii)*, *Trichoderma harziaum* strains T22, *Pseudomonas fluorescens* strain DSMZ13134 and *Bacillus velezensis* strain FZB42 have been characterized within the project.

Generally, *P. bilaii* is claimed to be able to improve P uptake by plants by enhancing the availability of P source for plants and increase thus plant growth. However, plant growth promoting effects have also been observed under P sufficient conditions and cold stress (Goméz-Muňoz et al., 2018). As reported by Gulden and Vessey (2000), *P. bilaii* inoculation induces changes in root growth (influences the root hair formation and promotion of root growth), whereas a positive effect on P uptake was not detected in this study. Sánchez-Esteva et al. (2016) confirmed the increased shoot and root growth biomass of wheat plants when inoculated with *P. bilaii* on a moderately acid soil with application of sewage sludge, whereas on calcareous soil, the plant growth promoting effect was detected only when no additional P fertilizer was added. Thus, the P mobilization effect seems to be very environment dependent. Gómez-Muñoz et al. (2018) advert to the alleviation of abiotic stress after inoculation with *P. bilaii* soils with high P levels. This positive effect on plants cultivated under cold stress disappeared when tested on low P soil. Therefore, the inoculation with *P. bilaii* in order to mitigate cold stress is recommended for plants grown on substrates with high soil fertility. In another study from Gómez-Muñoz et al. (2018), the effect of available P was tested on maize inoculated with *P. bilaii*. The application of available P in combination with other nutrients resulted in an increased root growth and an improved nutrient uptake by maize plants inoculated by P. *bilaii*. However, if only plant-available P with no addition of other nutrients was applied, the shoot and root growth promoting effects of *P. bilaii* disappeared. Therefore, the plant

growth promoting effects of *P. bilaii* in this case, could be most likely attributed to an increased root growth under the presence of available P and other nutrients in the soil.

There is evidence for beneficial effects on root growth of plants associated with the inoculation of the fungus *Trichoderma* sp. The auxin-mediated improved root growth induced by the fungus might be a result of hormonal signalling and the production of volatile organic compounds (VOCs) by the fungus in particular (Garnica-Vergaga et al., 2015; Lee et al., 2016). Arabidopsis seedlings treated with *Trichoderma* for instance, perform stimulation of lateral root development, which is a characteristic response on auxin-related processes (Sofo et al., 2011). Björkman (2004) observed a successful root colonization of *Trichoderma* and a faster root growth of treated maize plants, whereas the response to auxin remain unchanged. In contrast, Sofo et al. 2011 reported a significantly increased level of IAA in both shoots and roots of plants inoculated with *Trichoderma*, resulting in improved root and shoot growth of inoculated plants. This finding is supported by the result of Saber et al. 2017. The plant growth of sorghum was significantly improved after the inoculation of *Trichoderma harzianum* WKY1 and the production of IAA on tryptophan-free medium was detected as well. Apart from improved shoot and root growth of host plant, *Trichoderma* is also known to act hyperparasitically. It is able to parasite a range of other pathogenic fungi and reduce thus the soil-born fungi diseases. One example of this mycoparasitism is *Trichoderma* parasiting the hyphae of *Rhizoctonia solanii* (Harman et al., 2004). *Trichoderma* is also known to directly solubilize phosphorous from sparingly soluble sources. Further, it is also able to mobilize micronutrients from the soil under specific conditions (Altomare et al., 1999).

Pseudomonas sp. strain DSMZ 13134 is claimed to colonize plant roots and stimulate both plant growth and its pathogen-defence system, resulting in overall stronger and more stable plants and yield improvement (www.sourcon-padena.de). A successful root colonization of *Pseudomonas sp.* strain DSMZ 13134 was observed by various authors (Buddrus-Schiemann et al., 2010; Nkebiwe et al., 2017). Furthermore, this strain is known for its biocontrol properties, production of siderophores and mobilization of P from plant-unavailable sources (Nkebiwe et al., 2017). Enhanced shoot growth and improved yield in *Pseudomonas sp.* strain DSMZ 13134 treated barley plants were observed in pot and field experiments especially in low nutrient systems. *Pseudomonas sp.* strain DSMZ 13134 causes a reduction of pH in artificial growth media and is thus able to solubilize P from insoluble sources. The production of siderophores is an important tool of *Pseudomonas sp.* strain DSMZ 13134 for micronutrient mobilization. These mechanisms maybe of great relevance especially under low nutrient

supply, since there the nutrient supply can be increased in greater extend (Fröhlich et al., 2012). However, no plant growth promoting effects of DMSZ 13134 were detectable on low P soils or after application of sparingly soluble P sources as reported by Lekfeldt et al. (2016), Thonar et al. (2017) and Mpanga et al. (2019a) and there was no indication for P solubilisation under rhizosphere conditions (Mpanga et al. 2019b).By contrast, if *Pseudomonas sp.* strain DSMZ 13134 was applied with organic fertilizers such as composted animal manures, beneficial effects on plant growth were detectable (Thonar et al., 2017).

The *Bacillus velezensis* strain FZB42 is known to have biocontrol properties by induced systemic resistance (ISR) of the host plant induced by the release of different bacterial metabolites but also direct pathogen suppressive potential via secretion of surfactins (Borriss, 2015). This strain is also able to colonize plant roots successfully and might thereby positively influence plant growth and plant health. The inoculation of *Bacillus velezensis* FZB42 improved for instance cotton yield under low N supply dramatically (Husseini et al., 2012). *Bacillus velezensis* FZB24 increased total yield of lettuce plants as well (Shehata et al., 2016). The major modes of action behind the beneficial effects on plant growth might be the mineral solubilisation and the secretion of different phytohormones and enzymes (Borriss, 2015). The production of IAA of bacterial origin could be observed in different *Bacillus* species (Lebuhn et al., 1997). For instance, improved plant growth attributed to successful root colonization of *Bacillus velezensis* FZB42 and its tryptophan-dependent IAA synthesis inducing promotion of lateral roots was detected by (Idris et al., 2007, Ramirez and Kloepper 2010; Borriss et al., 2011; Mpanga et al, 2019b). There is also a significant interaction between the soil P status and the *Bacillus* inoculation. At high rate of phytate present in the soil, plant growth promotion and an improved P uptake occurred in plants inoculated with *Bacillus* even under low P conditions. The ability of *Bacillus* to synthetize phytase and degrade thus the phytate might ensure plant growth promotion even under limited P conditions (Idriss et al., 2002; Ramirez and Kloepper 2010). The form of P fertilizer also seems to be a crucial factor influencing the efficiency of this bacteria. If applied with organic fertilizers, mainly composted animal manures, the bacteria interact beneficially with the host plant and improve the use efficacy of organic fertilizers (Naveed et al., 2008; Thonar et al., 2017, Vinci et al. 2018 a, b, Mpanga et al., 2018). The successful establishment of *Bacillus velezensis* FZB42 in the rhizosphere and its root colonization, without having any durable impact on the rhizosphere microbial community could be an interesting attribute of this strain for the future investigations (Chowdhury at el., 2013; Eltlbany et al, 2019). *Bacillus velezensis* FZB42 also contributes to overall abiotic stress

tolerance via auxin-related, ROS scavenging or proline synthetizing pathways as described by Liu et al., 2017.

3.3.2 Microbial combination-products

Increasing evidence suggests superior performance of microbial combi-products based on two or more defined inoculant strains and/or non-microbial BS and their advantage towards single strain-based products is arising (Bashan, 1998). In some cases, the mixed inoculants may promote combinatory or even synergistic effects induced by their components. However, the additional synergistic effects of microbial combi-products are not achieved on a regular base. Their success strongly depends on strain specific properties and the rhizosphere competence and their ability for effective root colonization in competition with the indigenous microflora (Sarma et al., 2015). *Trichoderma harzianum* strain OMG16 is a root-endophytic fungus known for its plant-growth promoting properties. One of the main characteristics of this strain is an improvement of the total root length and the enlargement of the root surface especially in tomato and maize leading to better nutrient supply (J. Geistlinger pers. communication). The root elongation induced by Trichoderma strains might be attributed to the auxin-mediated processes in the plant roots (Björkman, 2004; Sofo et al., 2011). There is various evidence for improved plant growth when OMG16 was combined with Bacillus *velezensis* in a product Combifector A and Combifector B developed within the BIOFECTOR Eu Project. As observed by Mpanga et al., 2018, there was an increased shoot and root growth of tomato plants. The driver for the improved shoot growth was probably the improved P supply attributed to the increased total root length. P was a limiting factor in this case and the improvement of P supply brought an improvement of N and K supply in the OMG16 treated plants as well. An increased activity of Mn-Superoxide-dismutates (SOD) and an improved tolerance against oxidative stress as well as an improved cold-stress tolerance in treated plants treated with a combination of OMG16 and selected Bacillus strains (CombifectorA) was detected as well. Also, an increased level of anti-stress metabolites such as phenolics, flavonoids and proline was observed (Ahmed, 2017). *Trichoderma* species may suppress different plant diseases due to systemic or localized induced resistance, improved shoot and root growth of the host plant or the changes of the microbial communities on the roots of the host plant (Harman, 2006), which was also confirmed for the strain OMG16. The induction of local resistance in the plant roots and an induced systemic resistance (ISR) in the whole plant (so called bio-priming) of the immune system of the whole plant was observed and refer also to biocontrol properties of the fungus. There is evidence for synergistic effects between certain

species, such as the combination of: *Paenibacillus mucilaginosus* + *Bacillus velezensis*; *Bacillus* spp. + *Trichoderma* spp. or the combination of beneficial bacteria with organic substances such as algae extracts, humic or amino acids. Yusran et al., 2009 sees a big potential in the usage of combi-products and suggests a further research on the effects of products based on *Pseudomonas spp.*, *Bacillu*s spp. and Arbuscular Mycorhizza Fungi (AMF). Synergistic and beneficial effects of a combination of *Pseudomonas putida* and *Bacillus velezensis* towards a single application of these species were observed under drought stress in chickpea (Kumar et al., 2016). An inoculation with a combi-product based on *Pseudomonas putida*, *Sphingomonas*, *Azospirillum* spp. and *Acinetobacter* sp. increased shoot and root dry weight of plants under drought stress more dramatically than the inoculation of single strains (Romero et al., 2017). Improved nutrient uptake and shoot growth of common bean (*Phaseolus vulgaris*) was observed after the application of *Bacillus* sp., *Pseudomonas* sp. and *Rhizobium leguminosarum* (Kumar et al., 2016). A combination inoculation based on *Paenibacillus polymyxa*, *Pantoea agglomerans* and *Funneliformis mosseae* (belonging to AMF) enhanced yield of French bean (*Phaseolus vulgaris*) under field conditions (Chauhan and Bagyaraj 2015). An additional plant growth promoting *Rhizobium-Azospirillum* effect on the growth of bean plants was confirmed by Remans et al., 2008. The combination of *Bacillus megaterium*, *Arthrobacter* sp. and *Enterobacter* sp. increased the yield of wheat significantly (Kumar et al., 2014). Couillerot et al. 2013 describes the positive effect on maize growth of a three-component inoculant based on *Glomus*, *Azospirillum* spp. and *Pseudomonas* spp., whereas Walker et al., 2012 observed unexpectedly similar effect of this three-component combi product on maize under field conditions as compared to the inoculation with the single strains. The efficacy of another three-component microbial combi-product based on *Pseudomonas fluorescens*, *Bacillus megaterium* and *Azospirillum lipoferum* was tested on wheat plants. The three-component combi-product improved N and P nutrition of wheat plants significantly (El-Komy, 2005). The micronutrient uptake and yield of wheat were increased after the inoculation with three-component microbial combi-product based on *Bacillus* sp., *Providencia* sp. and *Brevundimonas* sp. under pot-experiment conditions (Rana et al., 2012). A significant increase of antioxidants (flavonoids, ascorbic acid etc.) was observed in seeds of pea after the inoculation with microbial combi-product based on *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Jain et al., 2014). An advantage of a microbial combi-product based on *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Bacillus* spp., towards single strains tested on the plant growth of *Withania somnifera* was detected by Rajesakar and Elango, 2011. However, (Borriss, 2015) is more critical about the efficacy of the products consisting of

several microbial strains. Its variable product quality may not always ensure the same beneficial effects on plant growth. When vegetative cells of gram-negative bacteria such as *Pseudomonades* or *Rhizobium* are mixed with spores of gram-positive *Bacilli,* the plant-growth promoting effects of such mixture are not really predictable. Therefore, further research in this field still remains needed.

3.3.3 Microbial consortia products

There is a still growing interest in mixed inoculants based on a large number of bacterial strains to combine different beneficial properties and increase the probability for synergistic interactions a further benefit for plant growth. The manufacturers of such products claim the additional positive plant growth-promoting effects mainly due to stimulated physical and biochemical activities of the various biostimulants, which may enhance some of their beneficial aspects such as nutrient mobilization. secretion of phytohormones or pathogen suppression (Bashan, 1998). Since microbes in soil environments usually do not act as single species but as members of complex interacting microbial communities, responses to ta given environmental situation usually occur also at the population level. Thus, they are able to adapt to different environmental conditions and initiate not only competitive but also beneficial interactions between the specific members of the population. Different microbes of the population are able to adopt different physiological functions which ensure the active life of the microbial population. This cross-talk between bacteria termed as “quorum sensing” and the specific ability to act as one organism and react efficiently to various biotic and abiotic stresses implicates that a consortium of PGPMs may provide the plants with multiple benefits under variable environmental conditions, performing more efficiently than a single strain-based inoculation (Nuti and Giovannetti 2015; Sekar et al., 2016).

However, the production of MCPs based on single strain fermentations is expensive. Therefore, the huge number of different microbial strains is frequently coming from mixed-culture fermentation or composting processes based on various organic substrates. This results in a complex mixture which may contain different aerobic and anaerobic microbes as well as many fermentation metabolites or different organic substances (Calvo et al., 2014). According to the so-called “auto-selection hypothesis” for these products plant growth promoting properties are not simply determined by the applied agents but rather the host plant under the specific rhizosphere conditions is selecting the most suitable PGPMs and BS components out of the inoculated consortium for establishing an efficient interaction in the rhizosphere.,. Those microbial species with inefficient performance in terms of rhizosphere colonization and plant

growth promotion, are outcompeted by more dominant and stable populations which colonize the rhizosphere more intensively under the given conditions. This should theoretically ensure a broader field of usage of the MCP and extend thus its "application window", since different species of the whole spectrum contained in the MCP will promote their advantage under specific conditions. This applies also to various biotic and abiotic stress conditions. Microbial strains adopting specific stress-tolerance traits may exhibit preferential proliferation under stress conditions and could thus ensure plant stress tolerance and improved plant growth also under challenging environmental conditions (Lopez-Cervantes and Thorpe, 2013; BIOFECTOR Final Report 2017.).

However, the definition and particularly the standardization of the content of these products remains challenging, since during the fermentation process a more or less undefined consortium of microbes is established. Since a single strain can exert one or several of the postulated beneficial effects of the mentioned categories, no clear biological distinction is possible. Therefore, the manufacturers of these products usually guarantee only for the presence restricted number of microbial species. The rest remains unspecified in detail. Because of the non-specific microbial composition of these mixed products, it is difficult for the scientist to evaluate these products and prove their effectiveness (Bajwa 2005).

One example in this category of inoculants are represented by the "Effective Microorganisms" (EM) were developed by a Japanese scientist Teruo Higa, who claimed a holistic approach towards sustainable plant nutrition with so-called "friendly microorganisms". The content of this product was described as a mixture of more than 80 species of microorganisms including different PGPMs, photosynthetic (*Rhodopseudomonas palustris*) and lactic acid bacteria (*Lactobacillus* spp.), *Actinomycetes*, yeasts, organic acids and amino acids as well as fermenting fungi (*Aspergillus, Penicilium*) in presence of organic wastes, molasses and a range of beneficial microorganisms thriven in the mixture as products of the fermentation process (Higa, 1994; Hu and Qi, 2013). The photosynthetic bacteria synthetize various amino acids and sugars from the carbon present in root exudates. Lactic bacteria produce lactic acid from sugars coming from the root exudates. Since lactic acid is known for its sterilization properties, it could suppress different soil pathogens and accelerate the decomposition of organic matter in soil. The bioactive substances such as hormones and enzymes released by yeasts could promote root growth. Further, the antimicrobial substances produced by yeasts could support plant health as well (Condor-Golec et al., 2007). Thus, various positive effects on plant performance, increased crop yield and crop quality, enhanced plant

health and soil fertility and tolerance against biotic stresses such as pathogens and diseases, independent of the respective environmental conditions are to be expected. EM can also be used as prophylactics or as a natural “soil medicine” ensuring a balanced environment in the rhizosphere towards improved plant growth and increased yields (Higa and Par, 1994). The EM are often applied together with organic fertilizers, mostly different organic composts (so called *Bokashi*) or manure with the target of an accelerated decomposition of organic wastes due to the EMs. The organic fertilizer is primarily fermented with the EM and then inoculated to the plants as described in (Yamada and Xu, 2001). He further describes the positive effects of the EM in combination with organic fertilizer depending on the quality of the fermented organic fertilizer, addition of molasses and the influence of pH. The possible mode of action of the microbes present in the fermented compost-bacteria mixture could be either direct, depending on the carbon source present in the organic fraction serving as energy source for the bacteria or indirect, as an impact of metabolites synthesized synthetized by microbes (phytohormones, growth regulators etc.). However, a long–term field study, conducted over four years with four crops within long term organic farming trials, claimed beneficial EM effects mainly based on the nutrient content of the product (Mayer et al. 2010). A meta study of Megali et al. (2015) reported species- specific differences in EM responsiveness of different crops and even negative effects due to stimulation of insect pests in maize.

Another MCP similar to the EM developed in Taiwan is composed of 733 promising microbial strains including P-solubilizers, cellulotic bacteria, N-fixers and many others. The study was carried out in order to establish a promising multi-functional biostimulant with a broad field of usage. Similar as in the case of EM, this MCP was inoculated to plants after a prior combination with compost. The results indicated an enhancement in shoot growth of celery and an increase in the colony forming unit (CFU) of the beneficial bacteria per gram rhizosphere soil (Young et al., 2004).

A commercial MCP product tested in the studies within this PhD thesis is a liquid mixture of beneficial microorganisms as well as fungi, yeasts, algae and different enzymes, polypeptides and lactic acid arising from the fermentation processes. This MCP is produced by the company Agrinos, USA on behalf the company EuroChem Agro, Mannheim, Germany responsible for the European market. Most of the microbes contained in MCP, which may be responsible for the beneficial effects on plants are derived from fertile soil samples and commercial sources. The producer takes the guarantee only for the presence of *Azotobacter vinelandii* and *Clostridium pasteurianum* in the product. So called secondary microorganisms such as

Azotobacter vinelandii; *Bacillus* spp. (e.g. *B. velezensis, B. megaterium, B. subtilis*); *Clostridium* spp. (e.g. *Clostridium pasteurianum*); *Lactobacillus* spp.; *Nitrosomonas* spp.; *Nitrobacter* spp.; *Pseudomonas* spp. (e.g. *Pseudomonas fluorescens*) and *Rhizobium* spp. as well as fungi with biocontrol properties such as *Trichoderma harzianum* or algae extracts based on *Ascophyllum nodosum* and *Arthrospira platensis* may occur in the product as well. Water and molasses is a carrier solution for this product. According to the (Lopez-Cervantes and Thorpe, 2013) patent information, each component of the consortium has its specific function to ensure the balance of the whole rhizosphere system. The "auto-selection" hypothesis arises here as well, since according to the rhizosphere conditions, different microbial populations should be proliferated and more active than others. Different active microorganisms such as associative or symbiotic N-fixers are responsible for nitrogen fixation, P-solubilizers and P-decomposers convert immobilized phosphorus into its bio-available form, while others provide enzymes for breaking down plant residues, such as C-decomposers which release celluloses and degrade thus complex compounds into sugars, alcohols and organic acids. Others secrete enzymes such as peptidases and phosphatases, which are responsible for N and P turnover in soil. Strains tolerant to different abiotic stresses occur as well. Different strains exhibit antibiotic action and biological competition for pathogens. They also produce enzymes downgrading cell walls of pathogens such as chitinases or lipases. Certain populations regulate the pH in the soil while others simply serve as C source for the rest of the consortium. The fermented yeast provides trace elements and free amino acids.

The metabolism of each group of the consortia is therefore very interdependent and a close symbiotic association of all the components is required in order to perform successfully in terms of plant growth promotion.

The appropriate usage of MCP is supposed to increase crop yields while reducing the conventional fertilizer and fungicide input, improving soil fertility and soil structure and establishing a balanced, sustainable agricultural system (Lopez-Cervantes and Thorpe, 2013).

Plant-microbial interactions and the role of the plant microbiome

The human microbiome and human gut microflora has essential metabolic functions relevant for human health (Gilbert et al., 2016). Interestingly, the essential importance of the soil microbiome and the rhizosphere plant-microbiome interactions for plant growth and health shows many similarities to the functions of the human microbiome. Accordingly, similar to

human therapeutic approaches whole microbiome transplantations have been demonstrated as successful strategies e.g. for suppression of pathogens (Kwak et al. 2018).

The great importance of plant microbiome for plant growth and plant health and the need for further investigations of the plant-microbe interactions in order to understand these processes more deeply for practical applications has been recognized already by the pioneer of rhizosphere research Lorenz Hiltner (1904). However, for the majority of rhizosphere microorganisms and their interactions with host plants, detailed knowledge is still missing (Berendsen et al., 2012; Mendes et al., 2013) but is expected to be largely increased due to the availability and rapid improvement of modern sequencing, metagenomics and metabolomics approaches

It is known that many biotic and abiotic factors such as climate and weather conditions, agricultural management, plant pathogens, developmental stage of the plant and plant species or soil type and soil structure crucially influence the diversity of microbial communities in the rhizosphere (Berg and Smalla, 2009).

Different agricultural practices (fertilization, mechanization, pesticides input, pH correction and many others) have a dramatic impact on the diversity of soil microbiome as well. It is known that the plant fitness strongly depends on the balanced plant-microbe interactions. The artificial decrease of microbial diversity in the rhizosphere can result in nutritional and health disorders in plants (Andreote and Pereira e Silva, 2017). Therefore, inducing directed shifts of microbial diversity and microbial composition correlating with enhanced plant health and improved yields in a balanced agricultural system adopting the use of PGPMs, (**Fig. 3**) could be a promising strategy in establishing a more sustainable crop production (Lupatini et al., 2017).

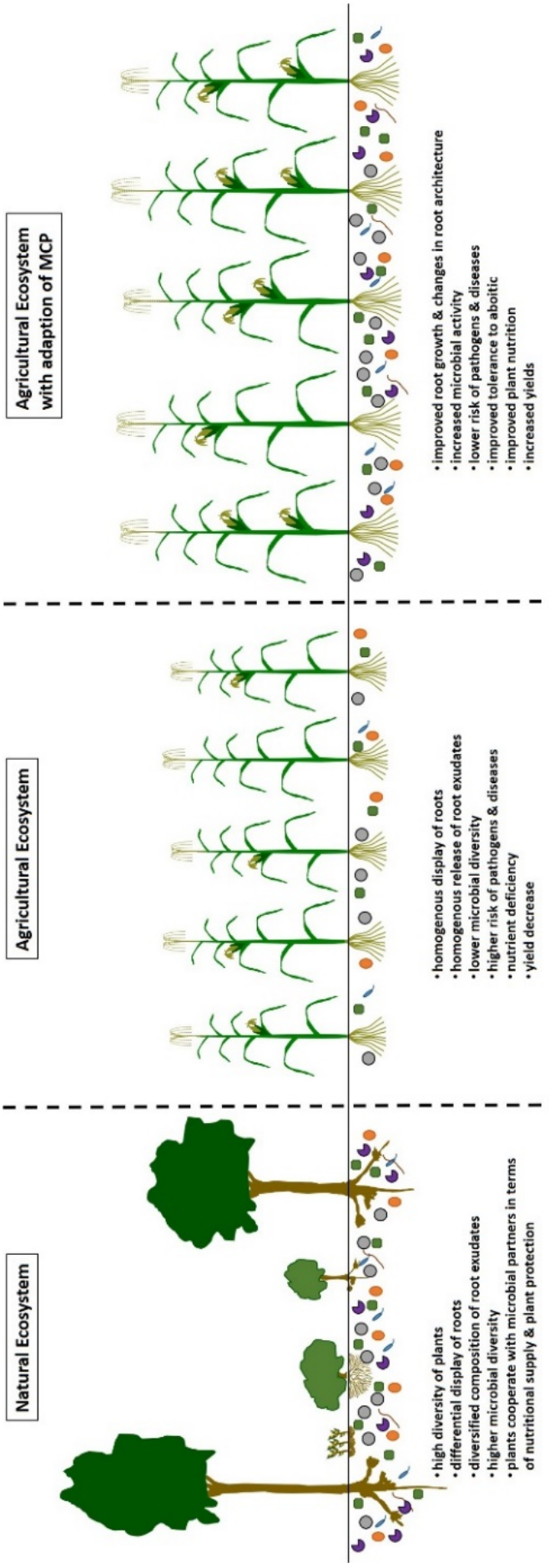

Fig. 3: Possible microbial interactions in different ecosystems (modified after Andreote et al., 2017)

3.4 Objectives and research questions

There is plenty of literature confirming the efficacy of specific single strain-based biostimulants and microbial combi-products. Also, information about advantages of certain combination products toward products based on single strains is available in some specific cases. However, the scientific literature systematically comparing the effects of microbial consortia versus single strain-based products is scarce and the underlying modes of action remain largely hypothetic. Further, there is a gap of scientific knowledge based mainly on the laboratory results with strictly controlled conditions and a confirmation of these specific beneficial effects of microbial consortia under real and practice relevant environmental conditions, confirming their potential advantage towards single strain-based products as often stated by the manufacturer of these products. The lack of understanding about the inter-relationships between plants and inoculated microbes and the microbe-microbe interactions as well as the difficulty in case of tracing and identification of the inoculated microbes in the practical field conditions remain a big challenge for further research in this topic (Sruthilaxmi and Babu, 2017). Accordingly, Bashan (1998), claimed that the relevance and significance of the impact of this co-inoculation with microbial consortia on plant yield must be further devised.

In the presented PhD thesis, these questions were addressed in model experiments and field trials with three different crops (maize, spring wheat, tomato) using a range of well-characterized single strain PGPMs and combination products in comparison with a commercial MCP inoculant. In a set of model experiments the MCP effects on nutrient acquisition in maize were characterized on soils with contrasting properties with respect to pH, soil structure, P availability, organic matter content and microbial activity and different forms and levels of N and P supply. The comparison of single strain inoculants, combination products and the MCP under real production conditions was performed in greenhouse and field production trials with wheat and tomato in Germany, Romania and Israel, addressing also interactions with the soil microbiome and the impact of environmental stress factors (P limitation, drought, heat).

The biostimulants of all three categories used in performed studies are listed in **Tab. 2**.

Tab. 2: Available manufacturer information and product description of the biostimulants used in performed studies

Product name, manufacturer	**Active ingredient**	**Expected effects on plants**
RhizoVital®42 TB, Abitep GmbH, Berlin, Germany	*Bacillus velezensis* strain FZB42 10^9 cfu g^{-1}	- Shoot and root growth stimulation and overall enhanced plant vitality - P solubilisation - Suppression of diseases
RhizoVital®42 TB +R41, Abitep GmbH, Berlin, Germany	*Bacillus velezensis* strain FZB42 *+ Bacillus simplex* strain R41 10^9 cfu g^{-1}	- Shoot and root growth stimulation and overall enhanced plant vitality - P solubilisation - Improved tolerance to abiotic stresses (e.g. cold stress)
ECAG 2920, EuroChem Agro, Mannheim, Germany	*Bacillus subtilis* $1*10^9$ spores ml^{-1}	- tolerance against abiotic stresses - Biocontrol properties
Proradix®WG, Sourcon Padena, Tübingen, Germany	*Pseudomonas* sp. strain DSMZ 13134 $5*10^{10}$ cfu g^{-1}	- Shoot and root growth stimulation and overall enhanced plant vitality - Improved availability of nutrients - Biocontrol properties - Suppression of pathogens due to intensive root colonization
Biological Fertilizer DC, Bayer CropScience Biologics GmbH, Malchow/Poel, Germany	*Penicillium bilaii* $1*10^9$ spores ml^{-1}	- Growth stimulation and enhanced vitality - P solubilisation - Micronutrients mobilization - Biocontrol properties - Induced systemic resistance against pathogens

CombiFector B, Anhalt University of Applied Sciences, Dr. Jörg Geistlinger, Bernburg, Germany (developed within the BIOFECTOR EU Project)	*Trichoderma harzianum* strain OMG16 $9*10^9$ spores g^{-1} *Bacillus velezensis* strain FZB42 $1*10^{11}$ cfu g^{-1} $ZnSO_4 * 7 H_2O$ $MnSO_4 * 1 H_2O$ Kaoline (mineral carrier matrix)	- Growth stimulation and enhanced vitality - Promoted root growth - Successful root colonization - Biocontrol properties - Pathogen suppression - Induced systemic resistance - Tolerance to abiotic stresses
MCP *I. composition****** Agrinos, Davis Ca, USA	Primary microorganisms: *Azotobacter vinelandii* $1.5 * 10^7$ cfu * ml^{-1} *Clostridium pasteurianum* $1.5* 10^7$ cfu * ml^{-1} Secondary microorganisms: *Clostridium spp.,* *Lactobacillus spp.,* *Rhizobium japonicum,* *Bacillus velezensis,* *Bacillus subtilis* SILoSil BS®, *Bacillus thurigiensis* SILoSil BT®, *Pseudomonas fluorescens sp.,* *Acetobacter spp.,* *Enterococcus sp.,* *Pediococcus sp.,* *Nitrobacter spp.,* *Nitrosomonas spp.,* *Nitrococcus spp.,* *Actinomyces, Micrococcus sp.,* *Streptomyces* Fungi: *Saccharomyces sp.,* *Penicillium sp.,* *Monascus sp.,* *Aspergillus sp.,* *Trichoderma harzianum* TRICHOSIL	- Shoot and root growth stimulation and overall enhanced plant vitality - Improvement of soil fertility - Yield improvement - Associative and symbiotic N_2 fixation - Phosphate solubilisation - Activation of C, N and P turnover enzymes - Regulation of soil pH - Enzyme and hormone secretion - Specific compounds serve as an energy source for occurring PGPM - Fermentation of carbohydrates - Secretion of secondary metabolites - Successful root colonization - Biocontrol properties - Pathogen suppression - Induced systemic resistance - Tolerance to abiotic stresses

	Algae: *Acophyllum nodosum,* *Arthrospira platensis*
MCP *II. composition*** Agrinos, USA; EuroChem Agro, Mannheim, Germany	Microbial composition: *Azotobacter vinelandii,* *Acetobacter pasteurianus,* *Bacillus sp.,* *Bacillus velezensis,* *Bacillus flexus,* *Bacillus licheniformis,* *Bacillus megaterium,* *Bacillus subtilis,* *Clostridium beijerinckii,* *Clostridium pasteurianum,* *Lactobacillus casei/paracasei,* *Lactobacillus buchneri,* *Lactobacillus delbrueckii,* *Lactobacillus vini,* *Oceanobacillus oncorhynchi,* *Paenibacillus chibensis,* *Paenibacillus cookie,* *Paenibacillus lautus,* *Pseudomonas sp.,* *Pseudomonas putida,* *Streptomyces griseus,* *Virgibacillus halophilus*

*MCP *I. composition* defined according to the Agrinos Patent Application (Lopez-Cervantes and Thorpe, 2013).

**MCP *II. composition* defined according to Agrinos / Eurochem Agro.

The following working hypotheses with the objective to further examine of the effects of MCP, identifying its modes of action and comparing the effects of MCP versus single strain-based products and combi products were addressed in this PhD. thesis:

1. Compared with single strain inoculants the microbial consortium product (MCP) provides higher flexibility under different environmental conditions and consequently exhibit a superior potential for plant growth promotion and increased yield.
2. The MCP induces direct P mobilization from sparingly soluble P sources.
3. The MCP is able to increase the availability of soil nutrients via increased enzymatic C, N and P turnover in the rhizosphere.
4. Improved spatial nutrient availability via improved root growth attributed to hormonal interactions with host plant is ensured by MCP.
5. The most suitable microbial populations of MCP will be activated and proliferated in the rhizosphere under specific conditions, while the less appropriate ones will be outcompeted. MCP inoculation can thus ensure improved plant growth and reproducibility of the effects under variable environmental conditions (auto selection hypothesis).

4 Functional characterization of nutrient acquisition in maize inoculated with microbial consortia product (MCP)

4.1 Microbial Consortia Stimulate Early Growth of Maize Depending on N and P Supply

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Abstract:

Adoption of microbial consortia as plant growth-promoting microorganisms (PGPMs) instead of single-strain inoculants is discussed as an approach to increase the efficiency and flexibility of PGPM-assisted production strategies. This study provides the functional characterisation of a commercial microbial consortia product (MCP) in a series of greenhouse experiments with maize on a silty-loam field soil (pH 5.9). A 60%-increased abundance of bacteria that could be cultivated after rhizosphere extraction was measured after MCP inoculation at the end of the 42-days culture period. MCP inoculation did not stimulate shoot biomass production of maize fertilised with nitrate, but growth improvement was recorded in combination with stabilised ammonium, especially with reduced phosphorus (P) supply. The MCP inoculant improved the acquisition of ammonium-N but also increased shoot-P. MCP inoculation stimulated root length development under reduced P supply with stabilised ammonium by 52%. This was accompanied by the increased auxin production capacity of rhizosphere bacteria. C-, N-, and P-turnover in the rhizosphere were little affected by the MCP inoculation, as deduced from the analysis of activities of extracellular soil enzymes. The findings suggest that the form of N supply is crucial for the efficiency of plant-MCP interactions.

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Microbial consortia inoculants stimulate early growth of maize depending on nitrogen and phosphorus supply

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Abstract: Adoption of microbial consortia as plant growth-promoting microorganisms (PGPMs) instead of single-strain inoculants is discussed as an approach to increase the efficiency and flexibility of PGPM-assisted production strategies. This study provides the functional characterisation of a commercial microbial consortia product (MCP) in a series of greenhouse experiments with maize on a silty-loam field soil (pH 5.9). A 60%-increased abundance of bacteria that could be cultivated after rhizosphere extraction was measured after MCP inoculation at the end of the 42-days culture period. MCP inoculation did not stimulate shoot biomass production of maize fertilised with nitrate, but growth improvement was recorded in combination with stabilised ammonium, especially with reduced phosphorus (P) supply. The MCP inoculant improved the acquisition of ammonium-N but also increased shoot-P. MCP inoculation stimulated root length development under reduced P supply with stabilised ammonium by 52%. This was accompanied by the increased auxin production capacity of rhizosphere bacteria. C-, N-, and P-turnover in the rhizosphere were little affected by the MCP inoculation, as deduced from the analysis of activities of extracellular soil enzymes. The findings suggest that the form of N supply is crucial for the efficiency of plant-MCP interactions.

Keywords: biofertilisers; root-associated microbiome; P solubilisation; acid phosphatase; plant-microbe interactions

The use of selected rhizosphere microorganisms with well-characterised beneficial properties as plant inoculants, commonly termed as "plant growth-promoting microorganisms" (PGPM), is discussed as a strategy with the potential to improve soil quality, plant health, nutrient acquisition and abiotic stress tolerance in cropping systems (Kloepper et al. 1991, Glick 2014). However, the expression of the desired effects under real rhizosphere conditions strongly depends on the rhizosphere competence of inoculants, i.e., their ability for compatible root colonisation of the host plant in competition with the indigenous microflora (Rajasekar and Elango 2011, Rana et al. 2012) and on the resistance against the various abiotic stress factors. To improve the flexibility of PGPM products, combined formulations based on multiple PGPM strains with complementary properties, are increasingly employed as so-called microbial consortia (Nuti and Giovannetti 2015, Sekar et al. 2016). These may also contain non-microbial biostimulants and stress-protective nutrients. Due to high production costs of single-strain combinations, frequently even less-defined microbial populations, originating from fermentation of various natural substrates, farmyard manure or composting processes are used as inoculants (Higa and Parr 1994,

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Hadar 2011, Lopez-Cervantes and Thorpe 2013). However, in these applications, standardisation of the composition and the possibility to achieve predictable results is even more challenging than in case of single strain inoculants.

The objective of the study was to investigate the performance and the modes of action of a commercial microbial consortia product (MCP), based on a mixture of selected beneficial fungal and bacterial strains and microorganisms originating from a fermentation process of organic substrates, further supplemented with seaweed extracts and discussed as a more targeted approach (Lopez-Cervantes and Thorpe 2013). Based on the physiological properties of the inoculants, complementary effects on processes mediating mineralisation of organic C, N and P sources in soils, N_2 fixation, P solubilisation and hormonal effects on plant growth, as well as pathogen suppression have been hypothesised as modes of action involved in the MCP-induced plant growth promotion (Lopez-Cervantes and Thorpe 2013). Unfortunately, it is not possible to identify the individual contributions of the various microbial strains to plant growth promotion and their potential interactions within complex consortia under real rhizosphere conditions. Therefore, the starting point of this study was a functional characterisation of the MCP product as a whole, with respect to the postulated beneficial properties (Lopez-Cervantes and Thorpe 2013). For an exemplary demonstration of the expected effects under environmental conditions characteristic for the maize rhizosphere, a set of pot experiments was conducted with the application of both nitrate and also ammonium fertilisers which are increasingly used for starter fertilisation in maize production systems. Moreover, recent findings suggest a beneficial effect of ammonium fertilisation on plant-PGPM interactions with microbial genera also used in the investigated consortium (Mpanga et al. 2018, 2019a,b). As indicators for the postulated beneficial MCP effects on processes with impact on nutrient availability in the rhizosphere (Lopez-Cervantes and Thorpe 2013) activities of marker enzymes involved in N, P and C cycling were determined in the rhizosphere soil with and without MCP inoculation (Baldrian 2009). To address the MCP-related root growth-promoting potential, the auxin production capacity of bacterial populations, re-isolated from the rhizosphere of inoculated and non-inoculated plants was determined at different time points of the culture period. Plate-counting assays with the re-isolated bacteria were conducted as an indicator for the root colonisation efficiency of the inoculants.

While the first part of the study exemplarily characterised the expression of potential plant growth-promoting properties of the MCP under rhizosphere conditions, the second part focused on the plant responses. The effects of the inoculants on shoot biomass production, root length development and the mineral nutritional status were evaluated in maize plants with different levels of N (nitrate *vs.* ammonium) and P supply.

MATERIAL AND METHODS

Plant cultivation. Greenhouse culture of maize (*Zea mays* cv. Jessy) was conducted on a silty loam field soil collected from the Ap horizon of a maize cultivation site in South-West Germany (Horb am Neckar, GMS coordinates: 48°26'39.23"N, 8°41'28.68"E): pH_{CaCl_2} 5.9; available P_{CAL}: 52 mg/kg soil (VDLUFA 1991); total N 0.15%; total C 1.1%. The soils were sieved with 2 mm mesh size, and the culture substrate was prepared as a 2:1 soil-sand mixture. Mineral nutrients were applied at different fertilisation levels (mg/kg soil): (*i*) (N140, P80, K150, Mg50), representing a standard full nutrient supply; (*ii*) reduced N/P fertilisation (N70, P0, K150, Mg50); (*iii*) reduced P fertilisation (N140, P0, K150, Mg50); (*iv*) an unfertilised control (0-Ctrl). Phosphate was applied as Ca $(H_2PO_4)_2$, K as K_2SO_4, Mg as $MgSO_4$ providing also sulfur in the sufficiency range for all treatments. Nitrogen fertilisation was performed as $Ca(NO_3)_2$ (calcinit; Yara International, Oslo, Norway) or $(NH_4)_2SO_4$ stabilised with the nitrification inhibitor DMPP (3,4-dimethylpyrazol-phosphate, NovatecSolub21; Compo Expert GmbH, Münster, Germany). Culture vessels contained 3 kg of the substrate, and the moisture content was regularly adjusted gravimetrically to 70% of the substrate water-holding capacity (WHC) throughout the culture period.

Application of MCP. A commercial microbial consortia product (EuroChem Agro GmbH, Mannheim, Germany) was used for inoculation. The MCP was based on bacterial and fungal strains including *Azotobacter vinlandii*, *Clostridium* sp., *Lactobacillus* sp., *Bacillus velezensis*, *B. subtilis* (SILo Sil® BS), *B. thuringiensis*, *Pseudomonas fluorescens*, *Acetobacter*, *Enterococcus*, *Rhizobium japonicum*, *Nitrosomonas*, *Nitrobacter*, *Saccharomyces*, *Penicillium roqueforti*, *Monascus*, *Aspergillus oryzae*, *Trichoderma harzia-*

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num T58 (TRICHOSIL®) and algae extracts from *Arthrospira platensis* (Spirulina) and *Ascophyllum nodosum* (Lopez-Cervantes and Thorpe 2013). For inoculation, a suspension of MCP 0.01325% (*w*/*w*) with non-chlorinated tap water was applied by soil-drenching close to the plants according to the instructions of the manufacturer (10 mL/plant) at 0, 14 and 28 days after sowing (DAS).

Plant growth and nutritional status. At the final harvest (42 DAS), the root systems were washed out of the soil substrate, and loosely adhering rhizosphere soil was collected by shaking and stored at –20 °C until further analysis. Root length was determined after digitalisation using the WinRhizo root analysis system (Regent Instruments, Quebec, Canada) and root and shoot dry matter was determined gravimetrically after oven-drying at 60 °C. For analysis of the P status, 250 mg of dried plant shoot material was subjected to 1.5 h microwave digestion at 1400 W (ETHOS.lab Professional Microwave System, MLS, Leutkirch, Germany) after 30 min extraction in 5 mL HNO_3 (conc.) 1:3, 3 mL H_2O_2 (30%) and 2 mL deionised water. Spectrophotometric determination of orthophosphate was conducted after the addition of molybdate-vanadate color reagent according to Gericke and Kurmis (1952) using a Hitachi U-3300 spectrophotometer (Hitachi Ltd, Tokyo, Japan). Total shoot N was measured by elemental analysis with a Vario Max CN macro-elementar analyser (Elementar Analysensysteme, Hanau, Germany).

Isolation of rhizosphere bacteria and auxin production assay. After final harvest at 42 DAS, bacteria from two grams of root samples with adhering rhizosphere soil were isolated into 50 mL of 0.1% protease peptone with two grams of sterile glass beads according to the method of Broadbent et al. (1971). Plate-count assays were performed after transfer to Standard 2 Nutrient Agar (Merck, Darmstadt, Germany) and Kings B Medium (Sigma-Aldrich, Germany for detection of fluorescent Pseudomonades (Naglitsch 1996), documented under UV light (354 nm). The auxin production potential of the bacterial isolates in 0.1% protease peptone was determined spectrophotometrically according to the method of Glickmann and Dessaux (1995). Bacterial dry biomass in the assay solution was estimated gravimetrically after 5 min centrifugation at 8 000 × g and subsequent oven drying of the bacterial pellet.

Enzymes involved in C, N, and P cycling in the rhizosphere. The activity of marker enzymes, mediating C, N and P cycling in the rhizosphere was assayed with fluorogenic substrates containing the fluorescence indicator 4-methylumbelliferone (4-MUF; Sigma-Aldrich, St. Louis, USA) according to Stemmer (2004). A microplate reader (Microplate Fluorescence reader FLx800, BioTek Instruments Inc., Winooski, USA) was used for monitoring the enzymatic hydrolysis of the MUF substrates for β-D-glucosidase (Glu, EC 3.2.1.21); L-leucin peptidase (LLpep, EC 3.4.11.1); L-tyrosin peptidase (LTpep, EC 3.4.11.1); cellulase (Cell, EC 3.2.1.21); xylosidase (Xyl, EC 3.2.1.37), acid (EC 3.1.3.2) and alkaline phosphomonosterase (EC 3.1.3.1) at 360/460 nm.

Experimental design and statistical evaluation. Experiments were arranged in a completely randomised design with five replicates per treatment. Prior to the analysis, outliers were eliminated according to Chromiński and Tkacz (2010). To ascertain significant differences, a one-way ANOVA analysis with a Tukey-test ($P \leq 0.05$) was performed. The results are presented as adjusted means ± standard deviations (SD). The SAS/STAT software package of SAS® 9.4 (2016) (SAS Institute Inc., Cary, USA) was used for statistical analysis.

RESULTS AND DISCUSSION

MCP effects on the abundance of rhizosphere bacteria. Re-isolation of cultivable bacteria from the rhizosphere of maize plants, using plate-count assays with standard 2 nutrient agar revealed a significant increase in total colony forming unit (CFU) of the inoculated plants by 60% (Figure 1) at six weeks after sowing (WAS). This point to a long-lasting rhizosphere effect, reflecting an increased abundance of MCP bacteria and/or a stimulatory effect on indigenous rhizosphere microbial communities induced by the repeated application of the inoculants, as recently shown also by Eltlbany et al. (2019). An interesting observation was the decline of fluorescent Pseudomonades induced by MCP inoculation, after plating of re-isolated bacterial communities on the selective Kings-B medium (Naglitsch 1996). Members of the genus *Pseudomonas* are known as efficient rhizosphere colonizers with plant growth-promoting but also pathogenic properties (Waschkies et al. 1994, Erlacher et al. 2014) and are included in the MCP inoculum. Therefore, a more detailed characterisation at the species level would be required to evaluate the potential consequences of declining *Pseudomonas* populations for the host plant. However, the observation exemplarily demonstrates a significant MCP

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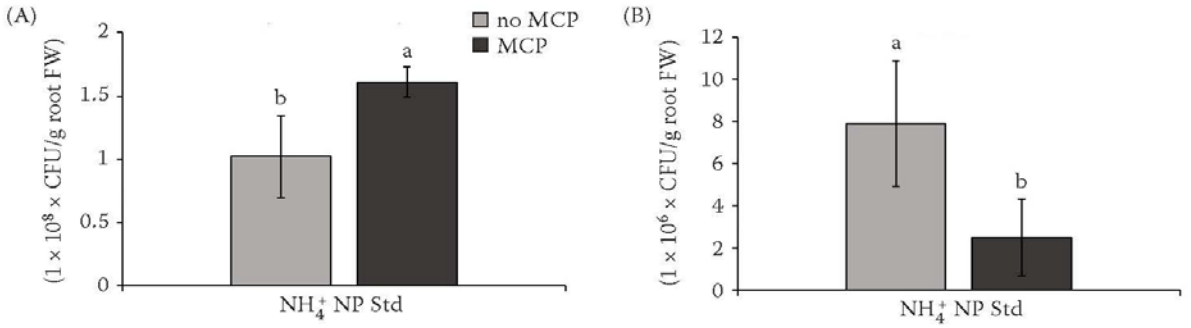

Figure 1. (A) Total cultivable rhizosphere bacteria on Std2 Medium and (B) colonies of fluorescent Pseudomonades cultivated on Kings B (KB) medium. Bacteria isolated from the rhizosphere of maize plants supplied with 130 mg ammonium N and 80 mg P per kg soil with and without microbial consortia product (MCP) inoculation. Data represent means and standard deviations of 5 replicates per treatment. Significant differences (Tukey-test, $\alpha < 5\%$) are indicated by different letters. CFU – colony forming unit; FW – fresh weight

interaction with the native soil microbiome as similarly shown in a more recent follow-up study, using an amplicon sequencing approach in a field experiment with tomato. In this case, the effects persisted even four months after the last inoculation, when no more changes in the abundance of the inoculated genera where detectable (Bradáčová et al. 2019a).

Marker enzyme activities for C, P, and N cycling in the rhizosphere. Based on the hypothesis that the MCP inoculants induce stimulatory effects on nutrient cycling in the rhizosphere (Lopez-Cervantes and Thorpe 2013), a functional characterisation was performed by measuring marker enzyme activities for turnover of C (β-glucosidase, cellulase, xylosidase), N (leucine and tyrosine peptidases) and P (acid and alkaline phosphatases) in rhizosphere soil samples according to Stemmer (2004), collected at 42 DAS. However, with the exception of a moderate decline in acid phosphatase activity by approximately 20%, and reduced cellulase activity, no inoculant effects were detectable (Table 1). Increased secretion of acid phosphatases is a typical response to P limitation by fungi and plant roots (Neumann and Römheld 2007), and the moderately declined activity may, therefore, reflect a slight improvement of the plant P status. The data do not support any direct involvement of the microbial inoculants in rhizosphere nutrient cycling, at least at the investigated sampling time. This is in line with recent follow-experiments, which showed significant stimulatory effects on the respective enzymatic activities in the maize rhizosphere only in a soil substrate with extremely low background activities of the respective enzymes, reflecting a low microbial activity due to long-term dry storage of the respective soil for more than 20 years. By contrast, on a freshly collected field soil, as similarly used in the

Table 1. The activity of rhizosphere marker enzymes for C, N, P turnover in the rhizosphere of maize plants fertilised with N in the form of Ca-nitrate (NO_3^-) *versus* DMPP-stabilised ammonium (NH_4^+) in comparison to an unfertilised control (0-Ctrl,) as affected by microbial consortia product (MCP) inoculation

Enzymatic activity (nmol × g/soil/h)		0-Crtl	NO_3^-		NH_4^+	
			no MCP	MCP	no MCP	MCP
C-turnover	β-D-glucosidase	601.45^{a}	490.73^{a}	526.97^{a}	667.35^{a}	549.09^{a}
	xylosidase	85.82^{a}	69.63^{a}	71.11^{a}	93.29^{a}	72.71^{a}
	cellulase	67.73^{ab}	88.10^{a}	63.26^{b}	57.90^{a}	76.50^{ab}
N-turnover	L-leucin-peptidase	441.70^{a}	359.30^{ab}	306.03^{b}	336.52^{b}	307.02^{b}
	L-tyrosin-peptidase	216.60^{a}	185.56^{ab}	151.01^{b}	164.85^{b}	170.97^{b}
P-turnover	acid-phosphatase	449.53^{a}	411.02^{ab}	333.92^{b}	411.15^{ab}	342.07^{b}
	alkaline-phosphatase	169.97^{a}	173.80^{a}	167.80^{a}	165.31^{a}	188.00^{a}

Presented data represent the means of five replicates. One-way ANOVA with Tukey test was performed. Different letters indicate significant differences between treatments ($P < 0.05$)

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present study, the inoculant effects were completely superimposed by high background activities of the rhizosphere marker enzymes exceeding the recorded MCP-induced changes by two orders of magnitude (Bradáčová et al. 2019b). This point to beneficial MCP effects on nutrient cycling and mineralisation preferentially expressed in heavily disturbed soil environments with limited microbial activities but not in fertile agricultural soils.

Auxin production potential of rhizosphere bacteria. Microbial production of auxins and molecules interfering with plant-hormonal signaling are among the best-documented features of PGPMs with beneficial effects on the root development of the host plants (Patten and Glick 2002, Ahmed and Hasnain 2010). Interestingly, rhizosphere bacteria re-isolated from MCP-inoculated maize plants with stabilised ammonium fertilisation revealed an increased auxin production potential as compared with the non-inoculated control, but exclusively in plants directly after the last MCP inoculation (28 DAS). This effect was lost at later stages of plant development at two weeks after the last inoculation (42 DAS) (Table 2). This is in line with earlier reports, demonstrating an increased auxin production potential detected for certain PGPM strains, such as of *Bacillus amyloliquefaciens* FZB42, *Pseudomonas* sp. DMSZ13134; *Pseudomonas putida* UB1 and *Acetobacter diazotrophicus* L1 (Patil et al. 2011, Bharucha et al. 2013, Mpanga et al. 2019a) supplied with ammonium instead of nitrate as mineral nitrogen source. These bacterial genera were also present in the MCP inoculant used in this study. Accordingly, Mpanga et al. (2019b) also reported beneficial effects of PGPM inoculants particularly during early growth of field-grown maize supplied with stabilised ammonium fertilisation, potentially related to the limited stability of the nitrification inhibitor. Consequently, nitrification and uptake of ammonium could explain the decline of beneficial effects on bacterial auxin production in the later stages of plant development. Similarly, Bradáčová et al. (2019b) reported root growth effects and expression of auxin-responsive genes in the root tissue induced by MCP inoculation of maize plants with stabilised ammonium fertilisation mainly during the first four weeks of the culture period, while the effects declined in later stages of plant development when inhibitory effects on nitrification were no longer detectable. However, the bacterial production of indole 3-acetic acid (IAA) has not only been related to the potential for stimulation of root growth but also with the ability for root colonisation, since IAA production mutants of various PGPR strains were also inefficient root colonisers (Spaepen et al. 2007). Therefore, the improved IAA production potential of bacterial populations in the rhizosphere of MCP-inoculated plants in response to ammonium supply may also contribute to improved root colonisation.

Table 2. Auxin production potential (relative values mg/microbial biomass) of rhizosphere bacteria isolated from maize roots inoculated with microbial consortia product (MCP) (with MCP) and non-inoculated control plants (no MCP) with nitrate or DMPP-stabilised ammonium fertilisation

Treatment		Harvest time			
		28 DAS		42 DAS	
		mean	SD	mean	SD
NH_4^+	no MCP	38.14[b]	17.69	272.88[a]	36.26
	with MCP	147.57[a]	27.88	330.94[a]	45.93
NO_3^-	no MCP	50.83[a]	23.01	361.51[a]	53.72
	with MCP	53.61[a]	53.00	386.59[a]	77.91

Data represent the means and standard deviation (SD) of five replicates. One-way ANOVA with Tukey test ($P < 0.05$) was performed. For each N form, different characters indicate significant differences between inoculated and non-inoculated plants. DAS – days after sowing

Effects on plant growth and mineral nutritional status. To assess potential benefits of the rhizosphere effects induced by MCP inoculation for plant growth and nutrient acquisition, a pot experiment was established comparing MCP responses of maize plants supplied with sufficient or reduced N and P supply and nitrate or ammonium as dominant nitrogen sources. Nitrogen was identified as the major limiting nutrient, and shoot growth was stimulated by 25% in comparison with an unfertilised control after the application of full N supply (140 mg N/kg soil) (Figure 2). This view is supported by the low N-status of control plants, indicating N deficiency (Figure 3A). The limited response to P fertilisation is in line with the moderate P availability of the investigated field soil (50 mg P_{CAL}/kg soil). Accordingly, the shoot P status (Figure 3C) at the end of the 42 days culture period reached 0.25–0.30% (*w*/*w*) even without additional P fertilisation, as a typical shoot P concentration under sufficient P supply (Campbell 2000). Nitrogen fertilisation increased both the N status (Figure 3A, B) and shoot growth, without significant differences between N forms (Figures 2 and 3).

Interestingly, MCP application improved shoot growth as compared with the non-inoculated controls in combination with ammonium supply but not with

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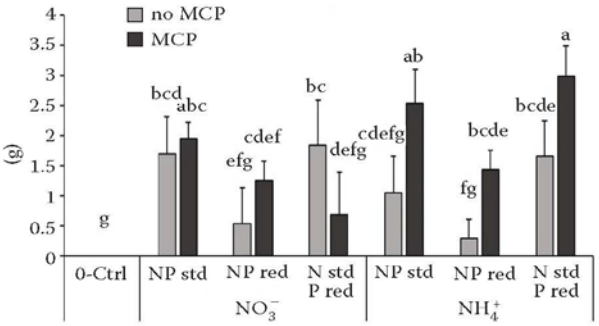

Figure 2. Increments in shoot dry biomass production of maize plants supplied with different levels of N (nitrate *vs.* ammonium) and P supply at 42 DAS (days after sowing) with and without microbial consortia product (MCP) inoculation as compared to the unfertilised control (0-Ctrl) (8.28 g dry matter (DM)). NP std = 140 mg N + 80 mg P/kg soil; NP red = 70 mg N + 0 mg P/kg soil; N std P red = 140 mg N + 0 mg P/kg soil. Data represent means and standard deviations of 5 replicates per treatment. Significant differences (Tukey-test, $\alpha < 5\%$) are marked with different letters above the bars

nitrate (Figure 2), as previously reported also for other inoculants based on strains of *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Trichoderma* (Mpanga et al. 2019a,b). The increased shoot biomass production of MCP-treated plants was independent of P fertilisation, suggesting that MCP inoculation mainly improved the acquisition of the limiting nutrient N, supplied in the form of ammonium. Similarly, Mpanga et al. (2019b) also reported improved acquisition of stabilised ammonium fertilisers in combination with PGPM inoculants. Even after a reduction of N supply by 50% without additional P application, plants treated with the MCP-ammonium combination had similar biomass as those receiving the full dose of nitrate and P fertilisation (Figure 2).

Although P was not a growth-limiting nutrient (Figure 3), a general trend for an increased P status, both with respect to shoot concentration, as well as in the P content, were observed in the MCP variants. The increase of the P concentration by the MCP treat-

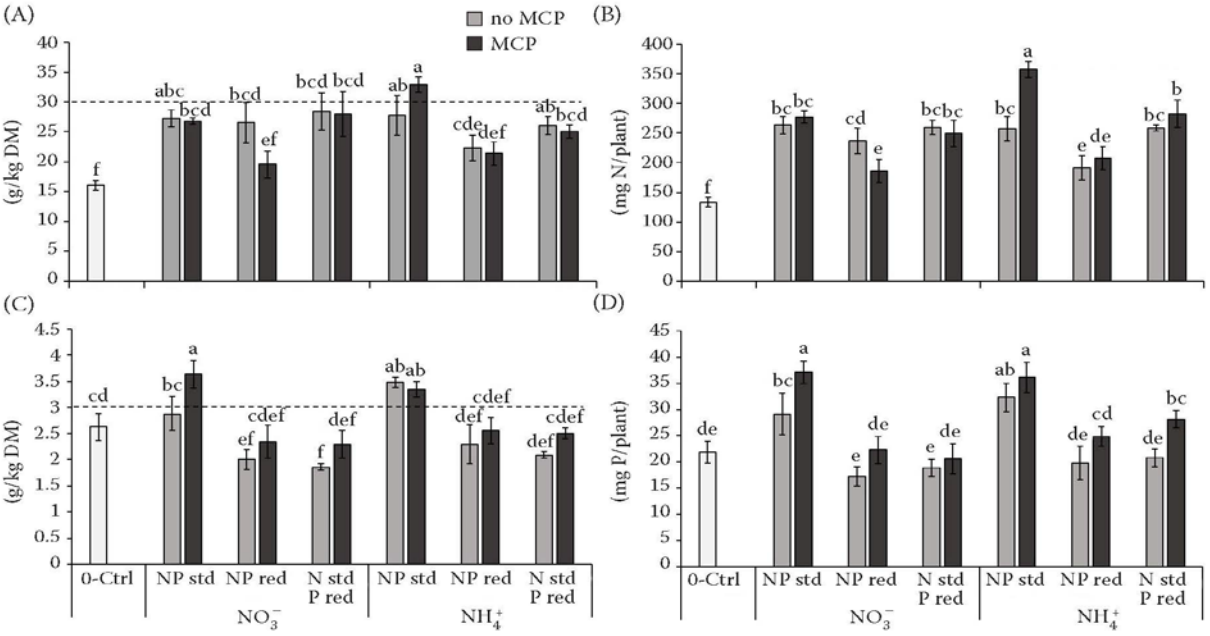

Figure 3. Concentrations and contents of N and P in the shoot tissue of maize plants with nitrate (nitrate) or stabilized ammonium fertilisation (ammonium) at 42 DAS (days after sowing) with and without microbial consortia product (MCP) inoculation. Values below the dashed lines indicate nutrient deficiencies, according to Campbell (2000). (A) N concentration in shoot tissue; (B) N content in shoot tissue; (C) P concentration in shoot tissue and (D) P content in shoot tissue. NP std = 140 mg N + 80 mg P/kg soil; NP red = 70 mg N + 0 mg P/kg soil; N std P red = 140 mg N + 0 mg P/kg soil. Data represent means and standard deviations of 5 replicates per treatment. Significant differences (Tukey-test, $\alpha < 5\%$) are marked with different letters

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ment, which was found in most treatments, except for the full ammonium-N and full P dose, indicates that the P utilisation within the plant changed. Furthermore, the P shoot accumulation was always increased by MCP inoculation, irrespective of the N-form supply (Figure 3C, D). The largest biomass was recorded with MCP inoculated plants with stabilised ammonium-N and reduced P fertilisation (Figure 2) and in this fertilisation regime, MCP inoculation was most beneficial for improving the P content of plants (Figure 3D). Direct effects of potential ammonium-induced rhizosphere acidification on P availability as described by Mpanga et al. (2019b), were not detectable in this study since the P status of the plants showed no N-form dependent differences in the absence of MCP inoculation (Figure 3C, D). Improved spatial acquisition of the native soil P *via* massively increased root length (+52%, Figure 4) is likely causal for the MCP effects under ammonium fertilisation (Table 2) and this effect can also improve the acquisition of other nutrients as recently shown by Mpanga et al. (2019a). This is also in accordance with the increased auxin production potential, detected for rhizosphere-bacterial populations of MCP inoculated plants. MCP inoculation increased shoot P concentrations also with full nitrate and P supply (Figure 3C, D) without any effect on root length (Figure 4). Thus, in this case, P acquisition of soluble P was stimulated by MCP inoculation *via* other, probably indirect mechanisms. A direct nutrient effect of the inoculum is unlikely since the effects were not detectable in all MCP treatments; the total P input of 0.19 mg/kg soil by the inoculum can be excluded as this was negligible.

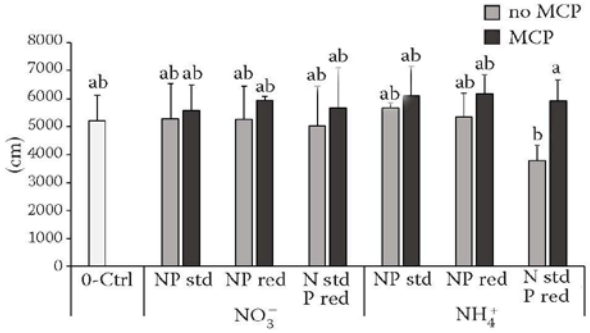

Figure 4. Total root length of maize plants with nitrate (nitrate) or stabilised ammonium fertilisation (ammonium) at 42 DAS (days after sowing) with and without microbial consortia product (MCP) inoculation. NP std = 140 mg N + 80 mg P/kg soil; NP red = 70 mg N + 0 mg P/kg soil; N std P red = 140 mg N + 0 mg P/kg soil. Data represent means and standard deviations of 5 replicates per treatment. Significant differences (Tukey-test, $\alpha < 5\%$) are marked with different letters

Taken together, the characterisation of the investigated MCP inoculant in a maize culture system revealed clear rhizosphere effects on the rhizosphere-bacterial abundance and composition, still detectable at the end of the 42 days culture period. Beneficial MCP effects on plant growth and nutrient acquisition were detected particularly in combination with stabilised ammonium fertilisation. The most intense growth stimulation was associated with significant MCP effects on root elongation for improved spatial nutrient acquisition under ammonium nutrition and increased auxin production of bacterial populations re-isolated from the rhizosphere of the respective plants. Similar results were recently reported for ammonium effects on maize plants inoculated with *Bacillus velezensis* (Mpanga et al. 2019b) as a bacterial species also present in the MCP inoculant. By contrast, there was no indication for longer-lasting MCP effects on nutrient cycling in the rhizosphere of the investigated soil. The results suggest that the form of N supply plays a crucial role in the expression of MCP effects and could offer a tool to improve the efficiency of plant-MCP interactions. However, the expression of MCP effects on different soils, different crops, the impact of variable environmental stress factors in the field and the relevance for real production conditions are aspects requiring further attention.

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4.2 Maize Inoculation with Microbial Consortia: Contrasting Effects on Rhizosphere Activities, Nutrient Acquisition and Early Growth in Different Soils

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Abstract:

The benefit of plant growth-promoting microorganisms (PGPMs) as plant inoculants is influenced by a wide range of environmental factors. Therefore, microbial consortia products (MCPs) based on multiple PGPM strains with complementary functions, have been proposed as superior, particularly under challenging environmental conditions and for restoration of beneficial microbial communities in disturbed soil environments. To test this hypothesis, the performance of a commercial MCP inoculant based on 22 PGPM strains was investigated in greenhouse experiments with maize on three soils with contrasting pH, organic matter content and microbial activity, under different P and N fertilization regimes. Interestingly, the MCP inoculant stimulated root and shoot growth and improved the acquisition of macronutrients only on a freshly collected field soil with high organic matter content, exclusively in combination with stabilized ammonium fertilization. This was associated with transiently increased expression of *AuxIAA5* in the root tissue, a gene responsive to exogenous auxin supply, suggesting root growth promotion by microbial auxin production as a major mode of action of the MCP inoculant. High microbial activity was indicated by intense expression of soil enzyme activities involved in C, N and P cycling in the rhizosphere (cellulase, leucine peptidase, alkaline and acid phosphatases) but without MCP effects. By contrast, the MCP inoculation did not affect maize biomass production or nutrient acquisition on soils with very little Corg and low microbial activity, although moderate stimulation of rhizosphere enzymes involved in N and P cycling was recorded. There was also no indication for MCP-induced solubilization of Ca-phosphates on a calcareous sub-soil fertilized with rock-phosphate. The results demonstrate that the combination of multiple PGPM strains with complementary properties as MCP inoculants does not necessarily translate into plant benefits in challenging environments. Thus, a better understanding of the conditions determining successful MCP application is mandatory.

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Maize Inoculation with Microbial Consortia: Contrasting Effects on Rhizosphere Activities, Nutrient Acquisition and Early Growth in Different Soils

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Abstract: The benefit of plant growth-promoting microorganisms (PGPMs) as plant inoculants is influenced by a wide range of environmental factors. Therefore, microbial consortia products (MCPs) based on multiple PGPM strains with complementary functions, have been proposed as superior, particularly under challenging environmental conditions and for restoration of beneficial microbial communities in disturbed soil environments. To test this hypothesis, the performance of a commercial MCP inoculant based on 22 PGPM strains was investigated in greenhouse experiments with maize on three soils with contrasting pH, organic matter content and microbial activity, under different P and N fertilization regimes. Interestingly, the MCP inoculant stimulated root and shoot growth and improved the acquisition of macronutrients only on a freshly collected field soil with high organic matter content, exclusively in combination with stabilized ammonium fertilization. This was associated with transiently increased expression of *AuxIAA5* in the root tissue, a gene responsive to exogenous auxin supply, suggesting root growth promotion by microbial auxin production as a major mode of action of the MCP inoculant. High microbial activity was indicated by intense expression of soil enzyme activities involved in C, N and P cycling in the rhizosphere (cellulase, leucine peptidase, alkaline and acid phosphatases) but without MCP effects. By contrast, the MCP inoculation did not affect maize biomass production or nutrient acquisition on soils with very little C_{org} and low microbial activity, although moderate stimulation of rhizosphere enzymes involved in N and P cycling was recorded. There was also no indication for MCP-induced solubilization of Ca-phosphates on a calcareous sub-soil fertilized with rock-phosphate. The results demonstrate that the combination of multiple PGPM strains with complementary properties as MCP inoculants does not necessarily translate into plant benefits in challenging environments. Thus, a better understanding of the conditions determining successful MCP application is mandatory.

Keywords: plant–microbial interactions; plant growth-promoting microorganisms; P solubilization; microbial consortia; ammonium; auxin-responsive genes

1. Introduction

The adoption of biostimulants (BS) based on bacterial and fungal inoculants or non-microbial bioactive compounds (e.g., humic acids, amino acids and peptides, chitosan, plant-, seaweed-, and

compost-extracts), has been discussed as a strategy to reduce the input of agrochemicals in crop production systems and related detrimental side effects on the environment [1,2]. Microbial and non-microbial BS may contribute to the mobilization of sparingly-soluble mineral nutrients, stimulate mineralization and nutrient cycling in the rhizosphere, promote root growth, and induce metabolic priming effects against biotic and abiotic stress factors in the target crops [3,4]. However, high variability and frequently limited reproducibility of the expected effects under real production conditions [5,6] suggests a strong impact of external factors, such as timing, dosage and mode of application, soil properties, fertilization management, environmental stress factors, interactions with the native soil microbiome, genotypic differences in responsiveness etc. To address this problem, the concept of consortia products based on different microbial strains and non-microbial BS with complementary properties was discussed as a strategy to increase the efficiency and the flexibility of BS-based production strategies under variable environmental conditions. Moreover, the composition of microbial consortia and non-microbial BS aims at the restoration of plant-beneficial, soil biological processes disturbed by soil degradation, intensive use of mineral fertilizers and chemical crop protection. This may apply for processes of nutrient cycling and mineralization, biological nitrogen fixation, nutrient mobilization and the pathogen suppressive potential in agricultural soils [4,7,8].

In this study, we aimed to characterize the performance of a commercial microbial consortia product (MCP) used as plant growth-promoting microorganism (PGPM) inoculant, based on carbon decomposers, providing easily available carbon sources for native rhizosphere biota and for microbial MCP strains involved in nutrient mineralization and biological N_2 fixation. Together with the activities of P-, and K-solubilizing inoculant strains and root growth promoters, an improved nutritional status of the host plants and the related rhizosphere-microbial communities was expected. Chitinase producers, PGPM strains belonging to the genera *Bacillus*, *Paenibacillus*, *Pseudomonas* were included to promote pathogen antagonisms and induce priming effects against abiotic stress factors [9]. Based on this hypothetic scenario, the function of the MCP product was characterized under real rhizosphere conditions in greenhouse experiments, using maize as a model plant with a limited potential for root-induced nutrient mobilization [10]. The preliminary results indicated that the expression of MCP effects on plant growth and nutrient acquisition was influenced by the dosage of N and P fertilizers but also by the form of N supply. The most intense expression of MCP effects on plant growth was observed in combination with ammonium fertilization, stabilized with a nitrification inhibitor (3,4-dimethylpyrazolephosphate, DMPP) and reduced P availability, which was associated with increased root length development. However, MCP inoculant did not change activities of C-, N-, and P-cycling enzymes in the rhizosphere of MCP inoculated plants [11].

In addition to the characterization of MCP performance depending on N and P supply [11], the effect of different soil types with limited P availability was addressed in the present study. Three soils with contrasting properties were selected for the experiments. To test the hypothesis of preferential MCP performance in disturbed soil environments [4,9], experiment 1 compared MCP performance on two low P soils with moderate P fertilization. To include an active soil microbiome, a clay loam with high organic matter content was freshly collected from the Ap horizon of a field site after grassland conversion. To represent a heavily disturbed soil environment with low microbial activity, a 20-year-stored, air-dried sandy loam substrate, characterized by low organic matter content with a low pH buffering capacity was selected. A functional characterization of soil microbial activities in the respective soils was performed by recording marker enzyme activities involved in C, N and P cycling in the maize rhizosphere. In experiment 2, the P solubilizing potential of the MCP inoculant under rhizosphere conditions was tested on a low P, calcareous Loess subsoil with Ca-phosphates as major mineral P form and low organic matter content. Without additional application of soluble P fertilizers, P acquisition of maize on this soil substrate would be almost exclusively dependent on mobilization of Ca-P. In all experiments, nitrogen was applied in the form of nitrate or DMPP-stabilized ammonium. Plant biomass production and nutritional status, root growth characteristics, expression of auxin-responsive genes in the root tissue, as well modifications in rhizosphere chemistry (rhizosphere

pH, marker enzyme activities involved in rhizosphere C, N and P cycling) were recorded to assess the effects of the MCP inoculants.

2. Materials and Methods

2.1. Soil Properties

Experiments were carried out on three different soils with contrasting properties. Soil 1 was a sandy-loam (pH_{CaCl2} 6.1) with low phosphate (P) availability (7 mg P_{CAL} kg^{-1}, (12 VFLUFA, 1991)) with low organic matter content (C_{org} 0.58%) and low microbial activity due to approximately 20-years storage under air dried conditions. By contrast, Soil 2 was a freshly-collected clay-loam field soil (pH 5.9) with limited P availability (20 mg P_{CAL} kg^{-1}) and high organic matter content (C_{org} 2.24%), to include an active soil microflora. The top soil was collected from the 0–30 cm Ap horizon at this field site. Soil 3 was a calcareous Loess sub-soil (pH 7.6) with low P availability (5 mg P_{CAL} kg^{-1}) and low organic matter content (0.16%), supplied with rock-phosphate (Rock-P) fertilization to assess the potential of the MCP inoculant for mobilization of sparingly soluble Ca-P in the rhizosphere. All the soils were air-dried, sieved with 2 mm mesh size and mixed with 30% (*w/w*) quartz sand to improve the soil structure. The specific chemical and physical soil properties are listed in Table S1.

2.2. Fertilization

Fertilization of the substrates was adapted according to soil properties, the experimental questions and the duration of the experiments. For soils 1 and 2, macronutrient supply (mg kg^{-1} soil) comprised: N: 140 as calcium nitrate (Yara Liva Calcinit, Yara International, Oslo, Norway) or DMPP-stabilized ammonium sulfate (NovaTec® Solub 21 (Compo Expert, Münster, Germany, with DMPP = 3,4-dimethylpyrazole phosphate as nitrification inhibitor), K: 150 as K_2SO_4; Mg: 50 as $MgSO_4$, and a moderate soluble P fertilization with P: 30 as $Ca(H_2PO_4)_2$. Soil 3 was fertilized with N: 100 as DMPP-stabilized ammonium sulfate (80%) and calcium nitrate (20%) to support P solubilization by ammonium-induced rhizosphere acidification [12]; K: 150 as K_2SO_4 and Mg: 50 as $MgSO_4$. Phosphate (P: 80) was supplied as sparingly-soluble Rock-P (Granuphos; 18% P_2O_5; Landor, Birsfelden, Switzerland) or as soluble $Ca(H_2PO_4)_2$ as a positive control treatment.

2.3. Test Plant and Culture Conditions

Maize (*Zea mays* L.) cv. Jessy (Advanta, Limagrain, Edemissen, Germany) was used as a test plant for all the experiments. Three seeds per pot were sown at depth of 1 cm and the soil surface was covered with a layer of fine quartz sand to minimize surface evaporation. For all experiments, the seeds originated from the same seed lot to account for potential differences in seed quality and the seed microbiome. After germination, thinning to one seedling per pot was performed and plant culture was conducted under greenhouse conditions with average air temperatures between 21 and 29 °C and 35–50% rel. humidity. Depending on the duration of the experiments between 28 and 41 days, substrate application comprised 2.4 kg (Soil 1), 2.9 kg (Soil 2) and 1.4 kg (Soil 3) per pot. Soil moisture was adjusted daily to 70% of the substrate water-holding capacity by gravimetric determination.

2.4. MCP Inoculation

The patented MCP inoculant [9] used in the experiment was provided by EuroChem Agro, (Mannheim, Germany) as a modified liquid formulation with 22 beneficial bacterial strains, including *Azotobacter vinelandii; Acetobacter pasteurianus; Bacillus amyloliquefaciens; Bacillus flexus; Bacillus licheniformis; Bacillus megaterium; Bacillus sp.; Bacillus subtilis; Clostridium beijerinckii; Clostridium pasteurianum; Lactobacillus casei/paracasei; Lactobacillus buchneri; Lactobacillus delbrueckii; Lactobacillus vini; Oceanobacillus oncorhynchi; Paenibacillus chibensis; Paenibacillus cookii; Paenibacillus lautus; Pseudomonas sp.; Pseudomonas putida; Streptomyces griseus; Virgibacillus halophilus* as declared ingredients. For inoculation, a suspension of MCP 0.01325% (*w/w*) with non-chlorinated tap water was applied by soil-drenching

close to the stems of plants (10 mL per plant for each inoculation step) depending on the duration of the experiments at 0, 14, 28 days after sowing (DAS) on Soil 1; 0, 7, 21, 34 DAS on Soil 2 and 0, 14, 21 and 34 DAS on Soil 3. Control plants (Ctrl) were treated with the respective amounts of non-chlorinated tap water.

2.5. Plant Growth and Nutritional Status

At final harvest, the root systems were washed out of the soil substrate, and loosely adhering rhizosphere soil was collected by shaking and stored at −20 °C until further analysis. Root length was determined after digitalization using the WinRhizo root analysis system (Regent Intruments, Quebec Canada) and root and shoot dry matter were determined gravimetrically after oven-drying at 60 °C. For analysis of the plant nutritional status, 250 mg of dried shoot material was subjected to 1.5 h microwave digestion at 1400 W (ETHOSlab Professional Microwave System, MLS, Leutkirch, Germany) after 30 min extraction in 5 mL HNO_3 (conc.) 1:3, 3 mL H_2O_2 (30%) and 2 mL deionized water. Spectrophotometric determination of orthophosphate was conducted after addition of molybdate-vanadate color reagent according to [13] using a Hitachi U-3300 Spectrophotometer, Hitachi Ltd., Tokyo, Japan). The concentrations of K and Ca were determined with an ELEX 6361 flame-photometer, Eppendorf, Germany). The concentrations of Mg were measured with an iCE 3000 Series Atomic Absorption Spectrometer (ThermoScientific, USA). Total shoot N was analysed with a Vario Max CN macro-elementar analyser (Elementar Analysensysteme, Hanau, Germany). N_{min} analysis of the bulk soil was conducted with 20 g samples of previously cold-stored (4 °C) soil, mixed with 80 mL of 0.0125 M $CaCl_2$ solution in a shaker for 1 h 200 rpm. After settling of the soil particles, the supernatant was filtered with 617 $\frac{1}{4}$ pleated filters (Machery-Nagel, Düren, Germany), The filtrates were stored in plastic bottles at −20 °C until analysis. The N_{min} analysis of the soil solutions was conducted with an AutoAnalyzer 3 (SEAL Analytical, Southampton, UK) for NO_3^--N and NH_4^+-N, respectively.

2.6. Expression of Auxin-Responsive Genes in the Root Tissue

The expression of auxin-responsive genes was tested using total RNA extracted from washed maize roots. After harvest roots were directly frozen in −80 °C, homogenized in liquid N_2 and total RNA was extracted using the innuPREP plant RNA Kit (Analytic Jena, Jena, Germany) following the manufactures instruction. Using the Quanti Tect Reverse Transcription Kit (Quiagen, Hilden, Germany), 1 µg of RNA was reverse transcribed into cDNA. Quantitative real time PCR for the auxin related genes IAA5 and PIN1c was performed using 15 ng cDNA per reaction. The reaction was performed in a CFX384 (Bio-Rad, Hercules, CA, USA) using the GreenMasterMix (2×) without ROX (Genaxxon bioscience GmbH, Ulm, Germany). Data were analyzed using the Bio-Rad CFX Manager 3.1 software. The housekeeping genes EF1a as well as beta-Tubulin were used as reference genes to normalize the expression data. Technical replicates were performed in triplicate. Expression of PIN1c was tested in one qPCR using three technical repetitions for two biological replicates.

2.7. Marker Enzymes as Indicators for C, N and P Cycling in the Rhizosphere

The activities of marker enzymes for C, N and P cycling in the rhizosphere soil was assayed with fluorogenic substrates containing the fluorescence indicator 4-methylumblliferon (4-MUF; Sigma-Aldrich, St. Louis, MO, USA) according to the method described by Stemmer [14]. A microplate reader (Microplate Fluorescence reader FL × 800, BioTek Instruments Inc., Winooski, VT, USA) was used for monitoring the enzymatic hydrolysis of the MUF substrates for L-leucin peptidase (EC 3.4.11.1), cellulase (EC 3.2.1.21), acid (EC 3.1.3.2) and alkaline phosphomonosterase (EC 3.1.3.1) at 360/460 nm.

2.8. Experimental Design and Statistical Evaluation

All experiments were arranged in a completely randomized design with five replicates and each one plant per treatment (Soil 1 and 2). Due to the extremely low P availability, the experiment on soil 3

comprised 10 replicates per treatment, since a weaker expression of MCP effects was expected under these conditions.

Statistical analysis was performed with SAS® 9.4 (SAS Institute Inc., Cary, NC, USA) statistical software by one-way ANOVA with Tukey test $p \leq 0.05$ for testing significant differences between treatments. Outliers were identified and removed based on the quartile method of [15].

An overview with a compilation of all experimental treatments is presented in Table S2.

3. Results

In the first experiment, potential MCP effects were compared on two moderately acidic low P soils with comparable pH (≈ 6) but contrasting properties with respect to soil texture, organic matter content and microbial activity. A first harvest was conducted between week 4 and 5 after sowing, when the first effects of the MCP inoculation became visually detectable (Figure S1).

3.1. Plant Growth

After a culture period of 4–5 weeks, shoot growth was superior on the sandy loam soil with low C_{org} and limited microbial activity, despite massively reduced root length compared to Soil 2. Furthermore, shoot accumulation of P was approximately doubled on Soil 1 and significantly increased in response to stabilized ammonium fertilization versus nitrate supply. However, root length even declined by approximately 60% in the variants with ammonium fertilization and MCP inoculation was entirely without effect on Soil 1 (Table 1, Figure 1).

By contrast, on the freshly collected clay-loam field soil (Soil 2), only the combination of stabilized ammonium fertilization with MCP inoculation significantly increased shoot biomass by 29% compared with the non-inoculated control with ammonium supply and even by 63% relative to nitrate fertilization. This was associated with a significantly increased root length by 32% and increased P accumulation (34%) exclusively in the MCP-ammonium combination.

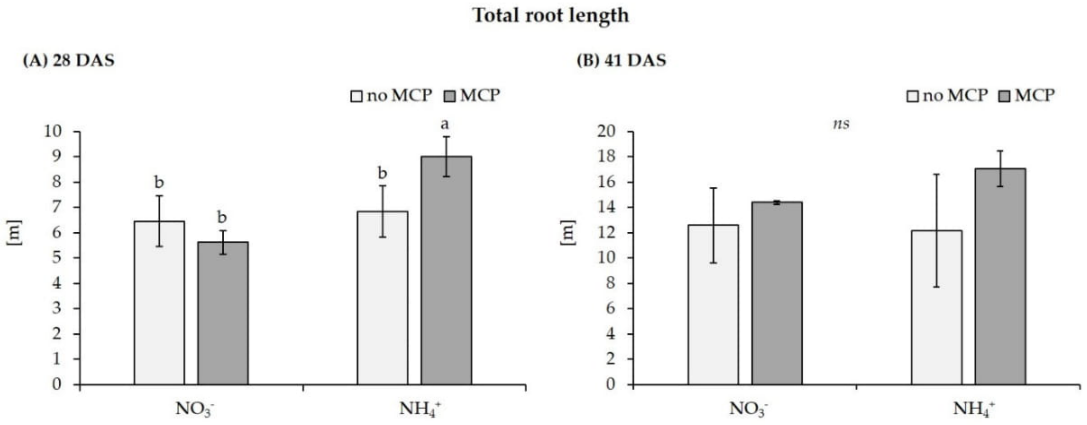

Figure 1. Effect of MCP inoculation on total root length of maize (cv Jessy) after a culture period of (**A**) 28 DAS and (**B**) 41 DAS on a clay-loam field soil (pH 5.9) with low P availability (Soil 2) supplied with moderate soluble P fertilization (30 mg P kg^{-1}) and N in form of Ca-nitrate (NO_3^-) or DMPP-stabilized ammonium (NH_4^+) Data represent means and SD of five replicates. A one-way ANOVA with Tukey test was performed. Different letters indicate significant differences between treatments ($p < 0.05$); ns = not significant.

Table 1. Shoot dry weight, total root length and P content in shoot tissue of maize plants (cv. Jessy) on two low P soils. as affected by MCP inoculation. Soil 1 (sandy-loam, pH 6.1, P_{CAL} 7 mg kg^{-1} soil) and Soil 2 (clay-loam, pH 5.9, P_{CAL} 20 mg kg^{-1} soil) receiving N fertilization in in form of Ca-nitrate (NO_3^-) or DMPP-stabilized ammonium (NH_4^+) and a moderate soluble P supply (30 mg P kg^{-1}). Data represent means and SD of five replicates. A one-way ANOVA with Tukey test was performed for data comparison. Different letters indicate significant differences between treatments ($p < 0.05$).

Plant Response	MCP Treatments	Soil 1		Soil 2	
		NO_3^-	NH_4^+	NO_3^-	NH_4^+
Shoot DW [g]	no MCP	3.32 ± 0.3 a	3.96 ± 0.2 a	2.09 ± 0.4 b	2.63 ± 0.3 b
	with MCP	3.37 ± 0.1 ab	3.90 ± 0.2 a	2.28 ± 0.5 b	3.4 ± 0.3 a
Total root length [cm]	no MCP	2718.9 ± 787.0 a	1048.3 ± 170.2 b	6454.6 ± 2954.1 b	6836.5 ± 4455.3 b
	with MCP	2325.3 ± 232.9 a	988.9 ± 448.6 b	5615.1 ± 132.0 b	9008.4 ± 1409.8 a
P content [mg $plant^{-1}$]	no MCP	6.05 ± 0.8 b	8.10 ± 0.4 a	2.89 ± 0.5 b	3.67 ± 0.4 b
	with MCP	6.50 ± 0.4 b	7.60 ± 0.6 a	2.95 ± 0.8 b	4.93 ± 0.4 a

3.2. *Rhizosphere Chemistry*

On both soils, the rhizosphere pH was higher with nitrate fertilization than with stabilized ammonium supply. The ammonium-induced rhizosphere acidification was particularly intense on the sandy loam (Soil 1), and reached a pH range between 4.6 and 5.1, indicating a low pH buffering capacity of the sandy substrate (Table 2).

Rhizosphere marker enzyme activities for C, N and P cycling were significantly higher on the freshly collected clay loam field soil as compared with the sandy loam (factor 4–16). This effect was particularly expressed for alkaline and acid phosphatase activities. As expected, on the clay loam with high endogenous microbial activity, MCP inoculation had no significant additional effects on enzyme activities. However, MCP application stimulated leucine peptidase activity by 36%, acid phosphatase by 39% and alkaline phosphatase by 28% on the sandy loam in combination with ammonium fertilization, while with nitrate fertilization a significant increase by 12% was recorded only for the activity of acid phosphatase (Table 2).

Table 2. Rhizosphere pH and rhizosphere-enzymatic activities of cellulase, peptidase and phosphatases as affected by MCP inoculation on soil 1 (sandy-loam, pH 6.1 P_{CAL} 7 mg kg^{-1} soil) and soil 2 (clay-loam, pH 5.9, P_{CAL} 20 mg kg^{-1} soil) receiving N fertilization in form of Ca-nitrate (NO_3^-) or DMPP-stabilized ammonium (NH_4^+) and a moderate soluble P supply (30 mg P kg^{-1}). Data represent means and SD of five replicates. A one-way ANOVA with Tukey test was performed for data comparison. Different letters indicate significant differences between treatments ($p < 0.05$). * indicates a significant difference between MCP inoculated (with MCP) and non-inoculated (no MCP) plants within the same N fertilizer treatments (Tukey test at $p < 0.05$).

	MCP Treatments	Soil 1		Soil 2	
		NO_3^-	NH_4^+	NO_3^-	NH_4^+
	no MCP	5.11 ± 0.1 a	4.60 b ± 0.1	5.79 ± 0.02 a	5.35 ± 0.04 b
	With MCP	5.42 ± 0.4 a	5.11 ± 0.2 a	5.79 ± 0.01 a	5.32 ± 0.06 b
Rhizosphere Enzymatic Activities [nmol g^{-1} soil h^{-1}]					
Peptidase	no MCP	42.01 ± 10.9	36.34 ± 6.2	160.28 ± 12.2	140.81 ± 6.6
	with MCP	47.92 ± 0.3	49.54 ± 1.6 *	142.60 ± 6.8	144.42 ± 11.9
Cellulase	no MCP	8.49 ± 0.9	7.67 ± 0.7	51.34 ± 5.5	52.28 ± 4.2
	with MCP	9.74 ± 1.0	8.15 ± 1.1	52.10 ± 1.1	48.19 ± 6.6
Acid Phosphatase	no MCP	111.76 ± 7.1	131.78 ± 22.2	964.96 ± 128.1	891.49 ± 28.0
	with MCP	125.62 ± 4.3 *	182.89 ± 19.5 *	948.89 ± 45.2	934.45 ± 125.2
Alkaline Phosphatase	no MCP	12.45 ± 3.6	7.0 ± 0.5	122.56 ± 18.8	113.96 ± 17.2
	with MCP	13.15 ± 2.3	8.98 ± 1.1 *	113.66 ± 17.3	127.16 ± 23.4

3.3. Plant-Nutritional Status

On both soils, shoot P concentrations dropped below the sufficiency threshold of 3 g kg^{-1} DM [16] (Table 3). However, analysis of shoot P accumulation revealed an increase by 27–34% induced by stabilized ammonium fertilization in comparison with nitrate supply (Table 4). Similar to the stimulation of root growth, a MCP effect on shoot P accumulation was exclusively detectable on the clay loam soil in combination with stabilized ammonium supply, with an increase by 34% compared with the non-inoculated control and even 71% compared with nitrate fertilization (Table 4).

On both investigated soils, shoot concentrations of N were low, but still in the sufficiency range (deficiency threshold 30 g N kg^{-1} DM) and the K status was sufficient (Table 3). Similar to the shoot P content, also N and K accumulation was significantly increased by 30% and 33%, respectively after MCP inoculation on the fresh clay loam field soil with stabilized ammonium fertilization (Table 4).

Shoot Ca and Mg concentrations were reduced under ammonium fertilization on both soils, as compared with nitrate supply and reached critical values close to the deficiency thresholds on the sandy loam soil (Table 3). Again, MCP inoculation induced a significantly increased shoot accumulation of Ca and Mg by 24 and 23%, exclusively on the silty loam soil with stabilized ammonium fertilization (Table 4).

Table 3. Shoot concentrations of mineral nutrients of maize plants (cv. Jessy) on two low P soils. as affected by MCP inoculation. Soil 1 (sandy-loam, pH 6.1, P_{CAL} 7 mg kg^{-1} soil) and Soil 2 (clay-loam, pH 5.9, P_{CAL} 20 mg kg^{-1} soil) receiving N fertilization in in form of Ca-nitrate (NO_3^-) or DMPP-stabilized ammonium (NH_4^+) and a moderate soluble P supply (30 mg P kg^{-1}). Data represent means of five replicates. A one-way ANOVA with Tukey test was performed for data comparison. Different letters indicate significant differences between treatments ($p < 0.05$).

Shoot Mineral Concentration (g kg DM^{-1})							
	N Forms	**MCP Treatments**	**N**	**P**	**K**	**Ca**	**Mg**
Soil 1	NO_3^-	no MCP	26.21 a	1.87 bc	51.19 a	3.94 a	1.99 a
		with MCP	25.25 a	1.82 c	49.22 a	3.95 a	1.99 a
	NH_4^+	no MCP	26.75 a	2.10 a	50.38 a	2.73 b	1.82 b
		with MCP	27.37 a	2.01 ab	49.55 a	2.75 b	1.74 b
Soil 2	NO_3^-	no MCP	37.54 a	1.38 a	36.75 b	4.92 a	2.71 a
		with MCP	35.42 b	1.24 b	36.61 b	4.81 a	2.65 a
	NH_4^+	no MCP	37.50 a	1.42 a	38.60 ab	4.15 b	2.33 b
		with MCP	37.73 a	1.45 a	39.88 a	4.00 b	2.12 c
Deficiency Threshold [16]			30.00	3.00	20.00	2.50	1.50

Table 4. Shoot accumulation of mineral nutrients in maize plants (cv. Jessy) on two low P soils. as affected by MCP inoculation. Soil 1 (sandy-loam, pH 6.1, P_{CAL} 7 mg kg^{-1} soil) and Soil 2 (clay-loam, pH 5.9, P_{CAL} 20 mg kg^{-1} soil) receiving N fertilization in in form of Ca-nitrate (NO_3^-) or DMPP-stabilized ammonium (NH_4^+) and a moderate soluble P supply (30 mg P kg^{-1}). Data represent means of five replicates. A One-way ANOVA with Tukey test was performed for data comparison. Different letters indicate significant differences between treatments ($p < 0.05$).

Shoot Mineral Content (mg $Plant^{-1}$)							
	N Form	**MCP Treatments**	**N**	**P**	**K**	**Ca**	**Mg**
Soil 1	NO_3^-	no MCP	88.62 a	6.05 b	163.7 b	12.67 ab	6.42 a
		with MCP	90.96 a	6.54 b	177.3 ab	14.60 a	7.28 a
	NH_4^+	no MCP	104.7 a	8.13 a	195.9 a	10.57 b	6.87 a
		with MCP	103.2 a	7.59 a	186.9 a	10.40 b	6.87 a
Soil 2	NO_3^-	no MCP	78.10 b	2.89 b	76.66 b	10.23 b	5.40 a
		with MCP	80.64 b	2.95 b	83.79 b	10.98 ab	5.84 a
	NH_4^+	no MCP	98.67 b	3.67 b	101.7 b	10.93 ab	5.87 a
		with MCP	128.1 a	4.93 a	135.5 a	13.57 a	7.21 a

3.4. MCP Effects on Root Growth and Expression Auf Auxin-Responsive Genes in the Root Tissue

The selective MCP-induced promotion of root growth on the clay loam field soil with stabilized ammonium fertilization was investigated in more detail. Root growth promotion by MCP and bacterial single strain inoculants in combination with stabilized ammonium supply have been similarly reported in recent studies and were associated with increased auxin production potential of bacterial populations re-isolated from the rhizosphere of inoculated maize plants [11,17]. This may pinpoint to root growth stimulation based on microbial auxin production.

As a more direct indicator, in this study, we investigated the expression of selected auxin-responsive genes in the root tissue of MCP-inoculated maize plants in relation to root growth responses. The *ZmAuxIAA5* gene was selected as a well-studied member of the auxin early response genes of the Auxin/Indole-3-Acetic Acid (Aux/IAA) family, that is rapidly up-regulated by external auxin supply [18]. The *ZmPIN1c* gene encodes an auxin efflux transporter involved in shoot to root translocation of auxins [19] and was selected as potential indicator of MCP effects on internal plant auxin homeostasis, independent of microbial auxin production. To address the longevity of the effects, measurements were conducted at two time points at 28 DAS and 41 DAS. For both time points, harvest was performed approximately one week after the last MCP inoculation.

A selective MCP-induced stimulation of root length was detected at 28 DAS on the fresh clay loam field soil with stabilized ammonium supply, but this effect disappeared at 41 DAS, showing only a gradual, non-significant increase. This was associated with a significant increase of *ZmAuxIAA5* expression at 28 DAS, but not at 41 DAS, whereas the expression of *ZmPIN1c* remained unaffected (Figure 2). Since MCP-induced root growth promotion was dependent on ammonium supply, we measured N_{min} in the remaining clay loam after harvest. Nitrate was the dominant N form detected in all variants. Compared with the initial N_{min} level of 144 mg kg^{-1} substrate, at 28 DAS, N_{min} was depleted by 58% in the nitrate variant and by 71% in the ammonium variant, where only 0.3% of the total N_{min} applied by fertilization remained in the ammonium form. At 41 DAS, nitrogen was depleted by >95% from the pots in all variants. In general, depletion of N_{min} was faster in the MCP variants as compared with the non-inoculated control, particularly under stabilized ammonium fertilization. Interestingly, ammonium N increased between 21 and 41 DAS, except for the ammonium variant lacking MCP (Table 5).

Table 5. Effect of MCP inoculation on total N_{min}, NO_3-N [mg kg^{-1} soil DM] and NH_4^+-N [mg kg^{-1} soil DM] after a culture period of 28 DAS and 41 DAS on a clay-loam field soil (pH 5.9) with low P availability (Soil 2) supplied with moderate soluble P fertilization (30 mg P kg^{-1}) and N in form of Ca-nitrate (NO_3^-) or DMPP-stabilized ammonium (NH_4^+) Data represent means and SD of five replicates. A one-way ANOVA with Tukey test was performed. Different letters indicate significant differences between treatments ($p < 0.05$).

	N Form	MCP Treatments	N_{min} Total	Soil NO_3^--N	Soil NH_4^+-N
28 DAS	NO_3^-	no MCP	60.45 ± 5.1a	60.45 ± 5.1 a	0 ± 0 c
		with MCP	45.46 ± 2.8 b	45.46 ± 2.8 b	0 ± 0 c
	NH_4^+	no MCP	42.58 ± 4.1 b	42.12 ± 4.1 b	0.46 ± 0.05 a
		with MCP	19.34 ± 0.5 c	19.26 ± 0.4 c	0.08 ± 0.04 b
41 DAS	NO_3^-	no MCP	2.73 ± 1.0 b	2.67 ± 0.9 bc	0.06 ± 0.02c
		with MCP	3.72 ± 4.4 b	3.69 ± 4.4 b	0.06 ± 0.04c
	NH_4^+	no MCP	7.09 ± 1.6 a	6.8 ± 1.4 a	0.29 ± 0.05b
		with MCP	2.42 ± 0.2 b	2.06 ± 0.1 bc	0.39 ± 0.04 a

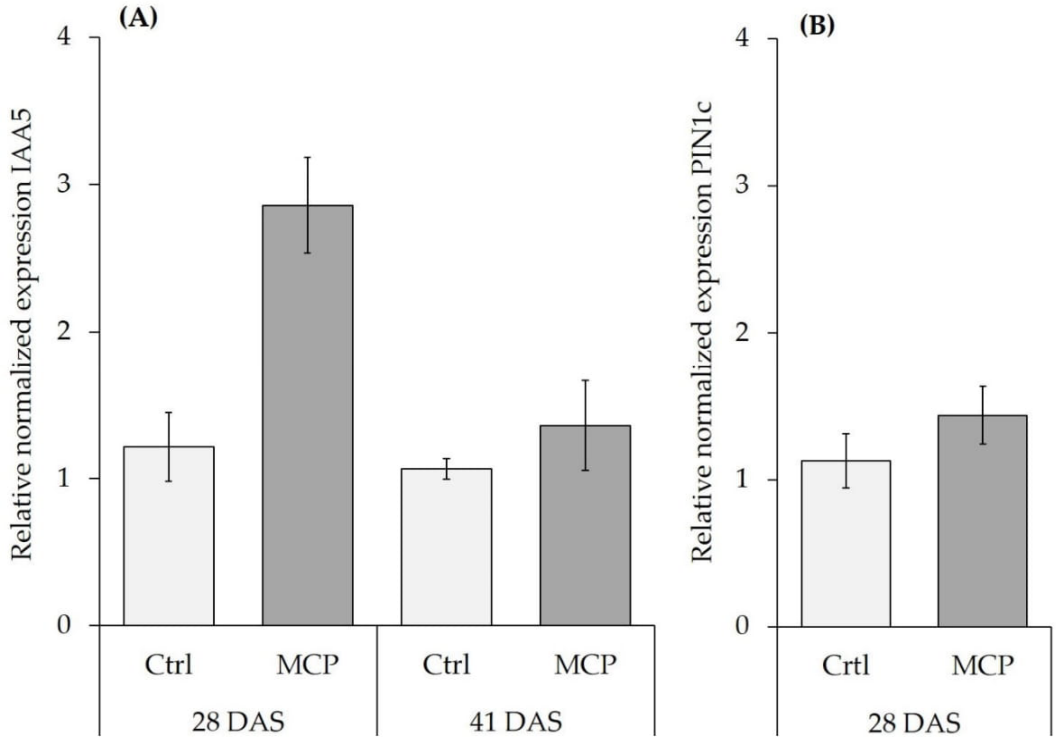

Figure 2. Relative normalized expression of auxin-related genes *ZmAux IAA5* (IAA5) and Zm*PIN1c* (PIN1c) in root tissue of control (Ctrl) and MCP inoculated (MC) maize plants grown on a clay-loam field soil (pH 5.9) with low P availability (Soil 2) supplied with moderate soluble P fertilization (30 mg P kg^{-1}) and N in form of DMPP-stabilized ammonium (NH_4^+). (**A**) IAA5 expression at 28 and 41 days after sowing (DAS). (**B**) PIN1c expression in plants at 28 DAS. Data for IAA5 represent means ± SD of two biological replicates, with nine technical replicates. For PIN1c data represent means ± SD of two biological replicates with three technical repetitions.

3.5. Phosphate-Solubilizing Potential of the MCP Inoculant

Experiment 2 was designed to assess the solubilizing potential for sparingly soluble soil P forms of the MCP inoculant. A calcareous Loess subsoil pH 7.6 with 23% calcium carbonate and extremely low P availability (P_{CAL} 5 mg kg $soil^{-1}$) and very low C_{org} was selected as culture substrate. Phosphate was applied as sparingly-soluble Rock-P or, with soluble $Ca(H_2PO_4)_2$ as a positive control. In the Rock-P variants, plant P acquisition was only possible after solubilization of Ca-P. Stabilized ammonium sulfate was applied as major N form to exploit the beneficial effects of ammonium on plant-MCP interactions and of ammonium-induced rhizosphere acidification on P solubility, similarly to experiment 1.

In the variants with Rock-P fertilization, MCP inoculation had no effect on shoot and root biomass production, root length and shoot P accumulation, as compared with the non-inoculated control (Figure 3A–D). Ammonium fertilization was not associated with rhizosphere acidification and the rhizosphere pH remained constant in all variants (pH 7.8). Although MCP inoculation had a marginal positive effect on the shoot P concentration, the P status remained severely deficient [16] and did not exceed 1 g kg^{-1} DM (Figure 3B). Only in the positive control with soluble P fertilization, the shoot P status reached the sufficiency range (3.3 g kg^{-1} DM), associated with an increase in shoot biomass by 450% (Figure 3A,B).

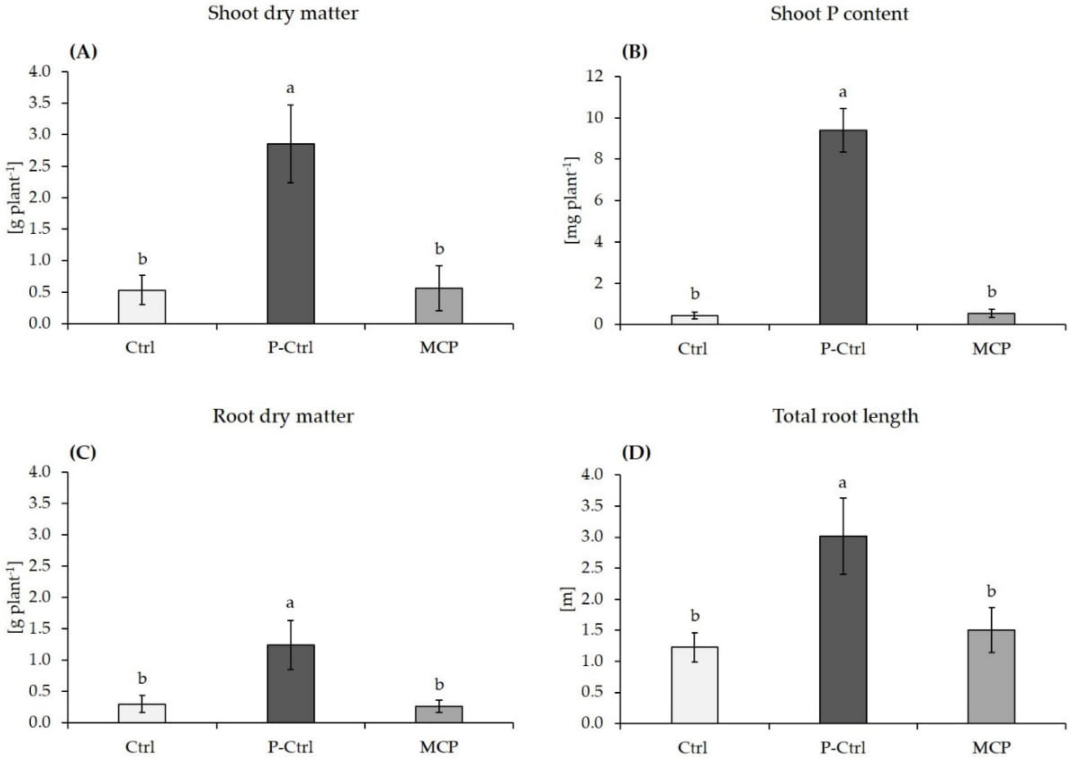

Figure 3. Shoot biomass (**A**), P content in shoot tissue (**B**), root dry matter (**C**) and total root length **D**)of maize plants (cv. Jessy) grown for 37 days on a calcareous Loess subsoil (pH 7.6) supplied with Rock-P (Ctrl, MCP treatment) or with soluble $Ca(H_2PO_4)_2$ as positive control (P-Ctrl). All plants received DMPP-stabilized ammonium (80 mg N kg soil^{-1}) and calcium nitrate (20 mg N kg soil^{-1}) as nitrogen sources. Data represent means and SE of ten replicates. A One-way ANOVA with Tukey test was performed for data comparison. Different letters indicate significant differences between treatments ($p < 0.05$).

4. Discussion

4.1. Effects of N-Form Supply on MCP Performance on Different Soils

The results of the present study confirm a beneficial effect of stabilized ammonium fertilization on the establishment of plant growth-promoting interactions, induced by MCP and other PGPM inoculants in plants with limited P supply (Table 1), as also previously reported by [11,17,20]. Synergistic interactions between ammonium fertilization and PGPM inoculation have been attributed to a range of different factors: (i) Root-induced rhizosphere acidification via proton extrusion in response to preferential ammonium uptake, which mediates the solubilization of acid-soluble soil P fractions [21], providing a P starter supply to the host plant under P-limited conditions [17,20]. This effect was detectable even on the slightly acidic soils (≈ pH 6) investigated in this study, where P was identified as limiting nutrient (Table 3; Table S1). The improved P-nutritional status promoted the establishment of beneficial plant-PGPM interactions in the rhizosphere. Accordingly, impaired rhizosphere establishment of PGPMs in extremely P deficient plants is well-documented [20,22–24]; (ii) Root growth promotion induced by the MCP inoculant has been observed in this study on the clay loam soil (Table 1). This improves not only P acquisition, but nutrient uptake in general (Table 4); [20,24]; (iii) ammonium fertilization can stimulate root hair development [17,25,26]; and (iv) ammonium nutrition stimulated auxin production of maize plants and various bacterial PGPM strains belonging to the genera *Bacillus*, *Pseudomonas* and *Acetobacter* [17,27,28]. Accordingly, also Bradacova et al. 2019 [11] found increased auxin production of bacterial populations that were re-isolated from the rhizosphere

of MCP-inoculated maize plants with stabilized ammonium supply. Strains of *Bacillus*, *Pseudomonas* and *Acetobacter* are also part of the investigated MCP formulation and therefore may be similarly involved in the increased auxin production of the bacterial populations in the maize rhizosphere after MCP inoculation [11].

Root growth stimulation via additional auxin supply by PGPM inoculants has been discussed as a major mechanism for plant growth promotion via microbes [29]. However, similar growth promoting effects have been demonstrated for bacterial quorum sensing signals or various volatile organic compounds (VOC's) of fungal and bacterial origin, which may interfere with auxin homeostasis in plants [29–31]. *AuxIAA5* gene expression is known to be rapidly induced by external auxin supply in roots [18]. In our study, *AuxIAA5* expression was upregulated on the silty loam field soil with ammonium fertilization one week after the last inoculation with MCP. This effect was restricted to 28 DAS, when increased root growth was apparent but was not detectable at 41 DAS. By contrast, expression of the *PIN1c* gene, that encodes an IAA efflux transporter, was not affected by MCP, supporting the hypothesis that the MCP-inoculant, but not the plant itself, supplied increased auxin levels to the root [11] *PIN1c* is activated by shoot-borne IAA and potentially involved in adventious rooting and acropetal auxin transport in the central cylinder [32]. By contrast, Garnica-Vergara 2016, [33] found an auxin-independent activation of *PIN* genes (*PIN1, PIN2, PIN3, PIN7*), associated with increased lateral root formation in *Arabidopsis thaliana* by 6-pentyl-2H-pyran-2-one (6-PP), a major bioactive VOC with potential cross-kingdom signaling functions emitted by *Trichoderma spp.* However, *Trichoderma* strains were not included in the MCP formulation tested in this study and accordingly PIN1c expression remained unaffected by MCP inoculation. Apart from root growth promotion, MCP inoculation also exerted stimulatory effects on rhizosphere marker enzyme activities for processes involved in mineralization of N (leucine peptidase) and P (acid and alkaline phosphatases), mainly in combination with stabilized ammonium fertilization (Table 2). In accordance with the hypothesis that MCP applications are particularly effective in disturbed environments by stimulating the recovery of beneficial microbial functions, positively linked to soil fertility [4,9], MCP effects on rhizosphere peptidases and phosphatases were detected exclusively on the sandy-loam soil that was stored for 20 years under air-dried conditions (Table 2) with very low C_{org} and limited microbial activity. In this soil, MCP inoculation stimulated peptidase and phosphatase activities, but the activity level still remained lower by magnitudes than in the freshly-collected clay loam field soil with an active soil microbiome (factor 4–16; Table 2). This underlines the generally low microbial activity in the sandy loam soil. Interestingly, higher peptidase and phosphatase activities in the rhizosphere of the MCP-inoculated maize plants in the sandy loam soil did not translate into any improvement of nutrient acquisition (Tables 3 and 4) and plant growth (Table 1). Substrate limitation for the enzymes due to the low organic matter content of the sandy loam soil (0.58%) may offer a possible explanation. In this context, co-application of organic fertilizers with MCP inoculants could offer a promising alternative, as recently shown for various single strain inoculants in combination with composted manures on low P soils in maize [23,34,35] and tomato [24]. By contrast, on the freshly-collected clay loam field soil, high background activities of cellulases, peptidases and phosphatases, reflecting a pronounced indigenous microbial activity, may have masked the comparatively small effects induced by the MCP-inoculants (Table 2).

The weak expression of MCP effects on marker enzyme activities involved in C, N and P cycling in the rhizosphere also indicates a limited direct or indirect impact of the MCP inoculation on these processes, e.g., by interactions with the soil microbiome. By contrast, a recent follow-up study demonstrated improved P acquisition of tomato after MCP inoculation combined with stabilized ammonium fertilization, in a drip-irrigated field experiment conducted in the Negev desert in Israel. Under these conditions, significant microbiome effects were detectable even three months after the last MCP inoculation [36]. An increased bacterial alpha-diversity at the rhizoplane was associated with a reduced abundance of *Sphingobacteria* known as salinity indicators and an increase in the population density of potentially plant-growth-promoting *Flavobacteria*. However, also in this case it was not clear

whether these effects must be regarded as a cause or rather as a consequence of the improved P status of the host plants, induced by MCP inoculation [36].

4.2. Limitations of Combined MCP Application with Ammonium Fertilizers

The beneficial MCP effects on plant growth were significantly promoted by combined application with stabilized ammonium fertilization (Table 1; [11]), but rapid microbial transformation of ammonium to nitrate in well-aerated agricultural soils may impede the practical use of this observation in agronomy. Therefore, the use of nitrification inhibitors seems to be mandatory to counteract rapid N turnover. However, nitrification inhibitors, such as DMPP, are active in soils only for limited time periods due to microbial degradation [37]. This explains the almost complete nitrification of ammonium already at 28 DAS on the clay loam soil. Accordingly, root growth promoting effects of MCP inoculation (Figure 1) and related upregulation of the auxin-responsive *AuxIAA5* gene in the root tissue of the maize plants (Figure 2) disappeared with increasing duration of the experiment at 41 DAS, even after repeated MCP applications. Longer-lasting effects of nitrification inhibitors may be expected under field conditions due to lower soil temperatures, lower rooting densities, and reduced rhizosphere microbial activities as compared with pot experiments. However, also under field conditions, plant growth-promoting effects of single strain inoculants and microbial consortia were most intensively expressed during early growth of maize plants [20]. Efficient field establishment is a critical factor for yield formation of maize particularly under unfavorable environmental conditions [38,39], but the establishment of longer lasting PGPM effects in agricultural production systems still remains a major challenge. Apart from limited stability of nitrification inhibitors in natural environments, repeated PGPM inoculations close to the root system for an efficient long-lasting root colonization are technically and economically challenging. This may be less difficult in horticultural production, were repeated application of PGPMs and stabilized ammonium fertilizers close to the roots is more easily integrated into widely-used drip irrigation or fertigation systems.

Apart from limited longevity, the expression of MCP effects was evidently also influenced by the respective soil properties. On the two moderately acidic soils (≈ pH 6), plant-growth promoting effects after MCP application were exclusively detectable on the fresh clay loam field soil with stabilized ammonium fertilization. However, the high endogenous microbial activities in this soil, reflected by high activities of rhizosphere enzymes, were not affected by the inoculation. As expected, increased rhizosphere enzyme activities involved in N and P cycling were detectable only in the MCP-inoculated variants of the sandy loam soil with a low intrinsic microbial activity but this did not translate into promotion of plant growth. By contrast, on the fresh silty loam field soil, MCP inoculation promoted root length development that resulted in a generally improved acquisition of macronutrients. An improved P-nutritional status could be partially attributed to increased P-solubilization due to moderate ammonium-induced rhizosphere acidification to pH 5.3–5.4 (Table 2). This was further supported by the root growth-promoting effects of the MCP inoculant (Table 1), leading to a larger acidifying root system for P mobilization and uptake. Improved P shoot accumulation due to ammonium-induced rhizosphere acidification was also detectable on the sandy-loam soil. However, due to a low pH-buffering capacity of the substrate, the rhizosphere pH in the ammonium variant dropped to pH 4.6 (Table 2), associated with inhibitory effects on root growth (Table 1). This was reflected in a 60% reduction in root length as compared with nitrate fertilization. At soil pH levels below 5.0, the risk of root growth inhibition induced by aluminium toxicity increases [40]. Moreover, the plant Ca and Mg nutritional status declined close to the critical thresholds (Table 3) as a consequence of the well-documented antagonistic effects of ammonium nutrition on uptake of cations, such as Ca and Mg [41]. This can lead to induced Ca and Mg deficiencies with detrimental effects on root growth [42–44]. Under these adverse conditions for root development, the root-growth promoting potential of the MCP inoculant was obviously not sufficient to exert any stimulatory effects on root growth and nutrient acquisition due to detrimental effects of limited root development on rhizosphere establishment of the inoculant.

By contrast, on the highly buffered and slightly alkaline subsoil (pH 7.6) with 23% calcium carbonate that was supplemented with Rock-P (experiment 2), ammonium fertilization failed to induce rhizosphere acidification, and the rhizosphere pH remained at pH 7.8 in all treatments. Phosphate was identified as growth-limiting factor, indicated by a 450% increased shoot biomass production and a P-nutritional status in the sufficiency range after application of soluble $Ca(H_2PO_4)_2$ instead of Rock-Phosphate as P fertilizer (Figure 3). However, even in combination with ammonium fertilization, the MCP inoculation had no beneficial effects on shoot biomass, root length development and P acquisition as compared with the non-inoculated control (Figure 3), and the plant P-nutritional status was severely deficient [16].These findings demonstrate that under the selected conditions, neither ammonium fertilization, nor MCP inoculation had any effects on solubilization of sparingly soluble Ca-phosphates, probably due to the high pH buffering capacity of the calcareous soil substrate. Moreover, similarly to the sandy loam soil, root development was severely inhibited in the severely P-deficient plants (Figure 3) as limiting factor for MCP root colonization. Instead the placement of homogenous application of the ammonium fertilizer may offer a solution to overcome this problem. Jing et al. [45] demonstrated that localized root proliferation in response to ammonium placement resulted in a significant decrease of rhizosphere pH of field-grown maize plants by two units even on alkaline soils with pH 8 in the ammonium depot zone. This effect was not detectable after broadcast application of ammonium fertilizers and was attributed to the high density of acidifying lateral roots in the application zone, increasing the intensity of rhizosphere acidification. This finally translated into improved P mobilization during early growth. Moreover, Nkebiwe et al. [46] demonstrated increased ammonium-induced root proliferation in the placement zone after PGPM inoculation of field-grown maize plants. These observations suggest that the combination of ammonium placement with PGPM inoculation could offer a strategy to overcome limitations of ammonium-induced P solubilization, otherwise limited by the high pH buffering capacity of the substrate.

4.3. Concluding Remarks

Based on the results of the present study, the beneficial effects of the selected MCP inoculant on plant growth and nutrient acquisition were strongly dependent on the form of nitrogen fertilization, soil properties and the plant developmental stage, as has been previously shown also for various single-strain inoculants [17,20,24]. Contrary to the initial hypothesis, compensating functions and preferential performance of the MCP inoculants in terms of plant growth promotion were not recorded on disturbed soils with limited nutrient availability, low microbial activity and low C_{org}. By contrast, MCP effects were preferentially expressed on a freshly-collected silty loam field soil with abundant, active microbes and moderate P availability.

Among the various modes of action discussed for beneficial PGPM effects on plant growth, stimulation of root growth, probably mediated via microbial auxin production, was identified as a major feature of the selected MCP inoculant. By contrast, stress factors, such as extreme P limitation (Figure 1) or soil acidity (Table 2) with inhibitory effects on root growth, already during the sensitive phase of MCP rhizosphere establishment, were associated with a limited expression of beneficial MCP effects on plant growth (Table 1; Figure 1). Under these conditions even multiple inoculant strains with differences in stress tolerance only have a limited advantage, as long as the stress conditions affect the ability of the host plant to support the establishment of a functional MCP interaction in the rhizosphere. Since this scenario is more likely in agricultural crops directly sown under field conditions as compared with greenhouse or nursery cultures, it remains a major challenge for practical applications. Although stimulatory effects of the inoculant on N and P cycling in the rhizosphere were detectable, these were not sufficient to translate into any benefits for plant nutrient acquisition or growth. Overall, there was no indication for solubilization of Ca-phosphates by the inoculant that could support plant P acquisition.

The findings of the present study demonstrated that similarly to single strain inoculants, MCP inoculants do not have a universal plant-growth promoting functions, but their successful application

strongly depends on various external factors. A better knowledge of the exact conditions required for the induction of beneficial effects on plant growth could provide a perspective for a more directed application of MCP-assisted production strategies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/7/9/329/s1, Table S1: Physical and chemical soil properties of three different experimental soils.

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5 Field performance of MCPs

5.1 Comparative evaluation of MCPs and single strain inoculants in tomato production with organic fertilization or placement of inorganic N/P fertilizers

Paper 3: Microbial Consortia versus Single-Strain Inoculants: an Advantage in PGPM-assisted Tomato Production?

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Abstract:

The use of biostimulants with plant growth-promoting properties, but without significant input of nutrients, is discussed as a strategy to increase stress resistance and nutrient use efficiency of crops. However, limited reproducibility under real production conditions remains a major challenge. The use of combination products based on microbial and non-microbial biostimulants or microbial consortia, with the aim to exploit complementary or synergistic interactions and increase the flexibility of responses under different environmental conditions, is discussed as a potential strategy to overcome this problem. This study aimed at comparing the efficiency of selected microbial single-strain inoculants with proven plant-growth promoting potential versus consortium products under real production conditions in large-scale tomato cultivation systems, exposed to different environmental challenges. In a protected greenhouse production system at Timisoara, Romania, with composted cow manure, guano, hair-, and feather-meals as major fertilizers, different fungal and bacterial single-strain inoculants, as well as microbial consortium products, showed very similar beneficial responses. Nursery performance, fruit setting, fruit size distribution, seasonal yield share, and cumulative yield (39–84% as compared to the control) were significantly improved over two growing periods. By contrast, superior performance of the microbial consortia products (MCPs) was recorded under more challenging environmental conditions in an open-field drip-fertigated tomato production system in the Negev desert, Israel with mineral fertilization on a high pH (7.9), low fertility, and sandy soil. This was reflected by improved phosphate (P) acquisition, a stimulation of vegetative shoot biomass production and increased final fruit yield under conditions of limited P supply. Moreover, MCP inoculation was associated with selective changes of the rhizosphere-bacterial community structure particularly with respect to Sphingobacteriia and Flavobacteria, reported as salinity indicators and drought stress protectants. Phosphate limitation reduced the diversity of bacterial populations at the root surface (rhizoplane) and this effect was reverted by MCP inoculation, reflecting the improved P status of the plants. The results support the hypothesis that the use of microbial consortia can increase the efficiency and reproducibility of BS-assisted strategies for crop production, particularly under challenging environmental conditions.

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Microbial Consortia versus Single-Strain Inoculants: An Advantage in PGPM-Assisted Tomato Production?

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Abstract: The use of biostimulants with plant growth-promoting properties, but without significant input of nutrients, is discussed as a strategy to increase stress resistance and nutrient use efficiency of crops. However, limited reproducibility under real production conditions remains a major challenge. The use of combination products based on microbial and non-microbial biostimulants or microbial consortia, with the aim to exploit complementary or synergistic interactions and increase the flexibility of responses under different environmental conditions, is discussed as a potential strategy to overcome this problem. This study aimed at comparing the efficiency of selected microbial single-strain inoculants with proven plant-growth promoting potential versus consortium products under real production conditions in large-scale tomato cultivation systems, exposed to different environmental challenges. In a protected greenhouse production system at Timisoara, Romania, with composted cow manure, guano, hair-, and feather-meals as major fertilizers, different fungal and bacterial single-strain inoculants, as well as microbial consortium products, showed very similar beneficial responses. Nursery performance, fruit setting, fruit size distribution, seasonal yield share, and cumulative yield (39–84% as compared to the control) were significantly improved over two growing periods. By contrast, superior performance of the microbial consortia products (MCPs) was recorded under more challenging environmental conditions in an open-field drip-fertigated tomato production system in the Negev desert, Israel with mineral fertilization on a high pH (7.9), low fertility, and sandy soil. This was reflected by improved phosphate (P) acquisition, a stimulation of vegetative shoot biomass production and increased final fruit yield under conditions of limited P supply. Moreover, MCP inoculation was associated with selective changes of the rhizosphere-bacterial

community structure particularly with respect to *Sphingobacteriia* and *Flavobacteria*, reported as salinity indicators and drought stress protectants. Phosphate limitation reduced the diversity of bacterial populations at the root surface (rhizoplane) and this effect was reverted by MCP inoculation, reflecting the improved P status of the plants. The results support the hypothesis that the use of microbial consortia can increase the efficiency and reproducibility of BS-assisted strategies for crop production, particularly under challenging environmental conditions.

Keywords: plant growth-promoting microorganisms (PGPM); biostimulants; microbial consortia; tomato production; organic fertilization

1. Introduction

The agricultural use of biostimulants (BS) based on microbial inoculants or bioactive natural compounds, originating, e.g., from plant, seaweed, and compost extracts or plant and animal based protein hydrolysates with plant growth-promoting and strengthening potential but without significant input of nutrients, has a long history [1,2]. Seaweed and gelatine-based. biostimulants are discussed to be a potential tool in terms of reducing the fertilizer and agrochemical inputs, which is often accompanied with negative environmental side effects [3–6]. Biostimulants could thus contribute towards more sustainable crop production. This is of particular importance for crop systems depending on intensive fertilizer input (e.g., vegetable production), associated with high risks of unwanted nutrient losses [7]. However, BS may also enable a more efficient use of organic and inorganic fertilizers based on materials and by-products of waste recycling [8–11], promoting concepts for the sustainable management of resources.

The commercial use of microbial BS in crop production was based initially on targeted selection of efficient single strain inoculants, starting with a first patent already in 1896 on Rhizobia to increase the atmospheric nitrogen fixation potential in leguminous plants [12]. Nowadays, numerous single-strain inoculants with biofertilizer functions are commercially available [1]. There is an increasing trend to use combination products based on microbial and nonmicrobial BS or microbial consortia, with the aim to exploit complementary or even synergistic interactions. Microbial consortia products (MCPs) are composed of compatible microbial strains with different modes of action to provide a broad spectrum of usage [13]. Strains of genetically diverse groups are selected, with the ability to adapt differentially to variations in environmental conditions, such as soil temperature, soil moisture, or soil pH [14]. However, due to high costs for single-strain production, frequently, strain combination products are at least partially replaced by less defined microbial populations, originating from fermentation of various natural substrates [13,15] or composting processes [16,17]. The concept behind these types of products is based on the assumption, that under variable environmental conditions, different members of the inoculated microbial communities are selectively activated by rhizosphere signals and ecophysiological responses of the host plant, to express their beneficial effects on plant growth. Examples comprise activation of phosphate solubilizing microorganisms in the absence of soluble P forms in soils, promotion of P mineralizers after supply of organic fertilizers or of chitinase-producing bacteria in response to proliferation of pathogenic fungi [13]. Various literature reviews claim that there is a clear trend showing the advantage of MCPs in comparison with single strain inoculants [1,14,18], but there are also contradictory reports [19] and particularly direct comparisons under real practice conditions are rare.

Based on the hypothesis of superior performance of microbial consortia over single-strain inoculants, in this study we present a comparative efficiency evaluation of a MCP versus a selection of fungal and bacterial single strain products and strain combinations with proven plant growth-promoting potential [11,20–23] under real production conditions.

Experiments were conducted under greenhouse and field conditions in two tomato production systems, characterized by different challenges with respect to type and amount of fertilizer supply, water availability, plant protection, and climatic conditions. In case study I, the effects of the different microbial inoculants were comparatively investigated during two seasons of commercial greenhouse tomato production in Timisoara, Romania with composted cow manure (nursery substrate), guano, hair, and feather meals (main culture) as major fertilizers frequently used in the local production practice. Case study II was conducted under more challenging environmental conditions in a drip-irrigated tomato production system in Ramat, Negev (the Negev Highlands), a desert region in Israel, on an alkaline sandy soil (pH 7.9) with very low phosphate availability (P_{Olsen}: 5 mg kg^{-1} soil DM), using microbial inoculants that were also investigated in case study I. The plants were supplied with different levels of mineral P supply (triple superphosphate), applied via band placement in combination with dicyandiamide (DCD)-stabilized ammonium sulfate to increase P availability on the alkaline soil. Microbial inoculants were applied via fertigation. In both case studies, the effects of single-strain inoculants on vegetative plant growth, yield formation, and fruit quality parameters were assessed in comparison with the consortium products.

2. Materials and Methods

2.1. Case Study 1: Large-Scale Greenhouse Tomato Production in Timisoara, Romania 2016/2017

2.1.1. Tomato Cultivation and Fertilization

The tomato (*Lycopersicum esculentum* L.) variety Primadona F1 (Hazera Seeds Ltd., Berurim M.P Shikmim, Israel) was used in the greenhouse experiment located at the horticultural research station of Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" Timișoara, Romania. The experiment was carried out under farmer's practice conditions. For preculture, tomato seeds were sown during February in plastic pots (1 seed pot^{-1}) containing 350–400 g of a nursery substrate mixture pH 7.3, based on 45% v/v composted cow manure, 30% v/v garden soil, 15% v/v peat, and 10% v/v sand (Supplementary Table S1). At phenological growth stage BBCH 51 in 2016, the nursery plants were transplanted for greenhouse culture into a clay loam Vertisol, pH 7.1 (Supplementary Table S1), preincorporated with an organic seabird guano (60%) and feather meal (40%) fertilizer (DIX 10N 10-3-3+1, 10% N, 1.3% P, 2.5% K, 0.6% Mg, Italpollina SpA, Rivoli Veronese, Italy) at the recommended dosage of 2.2 t ha^{-1}). Due to phytosanitary replant problems observed in 2016, in 2017 the nursery plants were transplanted into 10 L containers filled with a prefertilized clay peat substrate (SP ED63 T grob SM, 1kgNs+FE, Einheitserdewerke, Gebr. Patzer GmbH & Co. KG, Sinntal-Altengronau, Germany, N: 140 mg L^{-1}, P: 70 mg L^{-1}, K: 149 mg L^{-1}), plus 10% sand (v/v), pH 6.2. Additional organic fertilization was performed with a mixed hair/feather meal fertilizer (Monterra 13-0-0, 13% N, 0.22% P, MeMon BV, Arnhem, The Netherlands) at the recommended dose of 2 to 3 t ha^{-1} (=100 g $plant^{-1}$ in 10 L containers). In both years, supplementary foliar fertilization during the culture period according to the local practice was divided into 17 cumulative application rates with a total N, P, K application of 76.7, 1.8, and 3.3 kg ha^{-1}, respectively (details in Supplementary Table S2). The different types of commercial fertilizers used for the experiments comprised:

Lithovit® (Biofa AG, Münsingen, Germany), CO_2 foliar fertilizer: 77.9% $CaCO_3$, 8.7 % $MgCO_3$, 7.5% SiO_2, >0.25% Fe, >0.1% K_2O, >0.015% N, >0.015% P_2O_5, Al, S, >0.01% Mn, Cu, Zn.

CropMax® (Holland Farming B.V, Groenekan, The Netherlands): 0.2% N, 0.4% P, 0.02% K, 220 mg/L Fe, 550 mg/L Mg, 49 mg/L Zn, 35 mg/L Cu, 54 mg/L Mn, 70 mg/L B, 10 mg/L Ca, Mo, Co, Ni, aminoacids 2%, vitamins, enzymes, auxin, cytokinin, gibberellin.

YaraLiva Calcinit 15.5-0-0 (Yara UK Ltd., Grimsby, UK): 15.5% N (NO_3 14.4%, NH_4 1.1%), 19% Ca.

YaraVita® Universal Bio (Yara UK Ltd., Grimsby, UK): 8.5% N, 3.4% P_2O_5, 6% K_2O, 0.02% B, 0.1% Cu, 0.11% Mn, 0.003% Mo, 0.06% Zn.

Myr Calcium (Italpollina SpA, Rivoli Veronese, Italy): 3% organic N, 5% CaO, 18.5% organic C.

Myr Potassium (Italpollina SpA, Rivoli Veronese, Italy): 12% K_2O, 3% organic N, 11% organic C.

Plants were pruned after the development of 12 inflorescences (2016) and 10 inflorescences (2017), respectively. During the first eight weeks after transplanting, bumble bees (*Bombus* spp.) were deployed to facilitate pollination. Final harvest was conducted 15 weeks after transplanting.

2.1.2. Microbial Inoculation

Microbial biostimulants used in these experiments comprised Biological Fertilizer DC (Bayer CropScience Biologics GmbH, Malchow/Poel, Germany): active ingredient *Penicillium* sp. PK 112 (1×10^9 vital spores mL^{-1}); Proradix® WP (Sourcon Padena, Tübingen, Germany): active ingredient *Pseudomonas* sp. DMSZ 13134 (5.0×10^{10} colony forming units g^{-1}); RhizoVital® 42 fl. (Abitep GmbH, Berlin, Germany): active ingredient *Bacillus amyloliquefaciens* FZB42 (2.5×10^{10} spores g^{-1}), also referred as *Bacillus velezensis* FZB42; *Bacillus simplex* R41 (Abitep GmbH, Berlin, Germany, cold-tolerant strain, 2.5×10^{10} spores g^{-1}); and the microbial consortia product (MCP), (EuroChem Agro, Mannheim, Germany): declared active ingredients, twelve different beneficial bacterial strains including *Azotobacter vinlandii*, *Clostridium* sp., *Lactobacillus* sp., *Bacillus velezensis*, *B. subtilis* (SILo Sil® BS), *B. thuringiensis*, *Pseudomonas fluorescens*, Acetobacter, Enterococcus, *Rhizobium japonicum*, Nitrosomonas, and Nitrobacter, as well as fungi: Saccharomyces, *Penicillium roqueforti*, Monascus, *Aspergillus oryzae*, *Trichoderma harzianum* (TRICHOSIL®), and algae extracts from *Arthrospira platensis (Spirulina)* and *Ascophyllum nodosum* [13].

For application of the different BS products, suspensions were prepared freshly in nonchlorinated tap water: Biological Fertilizer DC (BFDC) 0.05% *w*/*w*, Proradix WP 0.02% *w*/*w*, RhizoVital 42 liquid formulation 0.04% *w*/*w* + *Bacillus simplex* (R41) 0.04% *w*/*w*, MCP 0.01325% *w*/*w*. Inoculation was performed after seedling emergence BBCH 12 (second primary leaf on main shoot unfolded, approx. 21 days after sowing) with 20 mL stock suspension of the respective microbial products per pot. Control plants (Ctrl) were treated with the respective amounts of non-chlorinated tap water. After transplanting into the greenhouse soil (2016), or into container culture (2017) at BBCH 51 (first inflorescence visible), each plant was supplied again with 250 mL of the respective BS suspensions. MCP treatments were performed by fertigation at a concentration of 0.03% *w*/*w*, as recommended by the manufacturer (details of the inoculation procedure are summarized in Supplementary Table S3). 2016: transplanting of tomato plantlets into greenhouse soil (18,940 plants ha^{-1}); 2017: transplanting of tomato plantlets into 10 L pots with peat substrate (22,000 plants ha^{-1}).

2.1.3. Plant Protection

Disease control against fungal pathogens was performed with various chemical fungicides: Mancozeb 80% (0.2% *w*/*w*); Metiram 80% (0.2% *w*/*w*); Propineb 70% (0.2% *w*/*w*); Folpet 80% (0.15% *w*/*w*), Clorotalonil 500 g/L (0.2–0.4% *w*/*w*); and Cu hydroxide + 50% Cu metallic (0.3% *w*/*w*). Control of insect pests was conducted by use of biocontrol systems (Biobest® Sustainable Crop Management, Westerlo, Belgium): plant protection system used for *Trialeurodes vaporariorum*: Encarsia system (*Encarsia formosa)* and Macrolophus system (*Macrolophus caliginosus*) + Nutrimac (of food eggs from Ephestia kuehniella); for aphids: Aphidius system (*Aphidius colemani* and *Aphidoletes aphidimyza*); for trips *Frankliniella occidentalis*: Swirskii system (*Amblyseius swirskii*) and Orius system (*Orius laevigatus*) and for spiders (*Polyphagotarsonemus latus* and *Tetranychus urticae*): *Phytoseiulus persimils* (details are summarized in Supplementary Table S4).

However, later in the season of 2016, the tomato plants showed symptoms of replant diseases, such as root rot induced by the soil-borne pathogen *Fusarium oxysporum* Schlecht f. sp. *radicis-lycopersici* Jarvis and Shoemaker. A high population density of larvae of the beetle *Agriotes lineatus* L. that can feed on the roots of tomato plants was recorded as well. Therefore, in the experiment conducted in 2017, the crop management was modified. To counteract soil-borne diseases, precultured nursery plants were not directly transplanted into the pathogen-affected greenhouse soil, but cultivation was performed in 10 L containers with a commercial clay peat substrate (see Section 2.1.1).

2.1.4. Experimental Design and Statistical Evaluations

The experiment was arranged in a randomized block design with four replicate blocks, each block comprising four treatment plots. The size of individual treatment plots was 8 m × 3.3 m (26.4 m^2) with 50 plants per plot. Plants were arranged in five rows per plot (10 plants per row). The distance between rows was adjusted to 1.5 m. The distance between plants within the rows was 33 cm. The total size of the experimental area with four variants in four replicates was about 634 m^2. Treatment variants comprised Ctrl (Control, no BS treatment), BFDC (Biological Fertilizer DC), Proradix (Proradix WP), FZB42 + R41 (Rhizovital® 42 liquid formulation + *Bacillus simplex* R41), and MCP (Microbial Consortia Product, EuroChem Agro, Mannheim, Germany). A two-way ANOVA with a Tukey test ($p \leq 0.05$) to ascertain significant differences was performed using the SAS Software package 9.4, Institute Inc., Cary, NC, USA.

2.1.5. Pre- and Postharvest Analyses

During the nursery phase, 21 days after the first application of the BS products, scoring of plant height and total leaf area (measured by a leaf area meter device) were determined for eight replicate plants per treatment group. Plant performance after transplanting was characterized for 30 plants per plot in terms of cumulative fruit yield ha^{-1}, mean weight per fruit, fruit biomass production per plant, fruit size distribution (three quality classes: II, I, and extra), and seasonal yield distribution during three months of harvest (June, July, and August), as relevant marketing factors.

2.2. Case study 2: Drip-Irrigated Field Production of Tomato (ARO Research Center), 2017

The field experiment was conducted in Ramat Negev, Israel on a sandy soil (96% sand), with low available P_{Olsen} (5.5 mg kg^{-1}), very low organic carbon (0.08%), and alkaline pH_{CaCl2} 7.9 (Supplementary Table S1). No precipitation occurred during the vegetation period as usual in the dry summer months of warm desert climates. Air temperature, relative humidity and radiation intensity are presented Supplementary Figure S1. The effects of BS applied as single-strain inoculants, as a combination product and a microbial consortium (MCP) were investigated in fertigated tomato plants with different levels of P supply applied by band placement 20 cm width and 30 cm depth along the row.

2.2.1. Tomato Cultivation and Fertilization

Tomato seeds (*Lycopersicum esculentum* L., var. Smadar, Hazera Seeds Ltd., Berurim M.P Shikmim, Israel) were sown on 10 March 2017 in a commercial nursery (Hishtil, Israel) into seedling trays containing Perlite medium (Agrekal Habonim Ind., Hof Hacarmel, Israel). During the nursery period, the seedlings were irrigated with above canopy sprinklers; irrigation was performed several times every day and in excess to allow drainage and to minimize water stress. Nutrients were delivered through the irrigation water. The concentrations of the macronutrients N, K, Ca, and Mg in the irrigating water: N: 50 mg L^{-1} (30% of N-NH_4); P: 13 mg L^{-1}; K: 62 mg L^{-1}; Ca: 6–80 mg L^{-1}; and Mg: 24 mg L^{-1}. Micronutrients were supplied at concentrations of Fe: 1 mg L^{-1}; Mn: 0.5 mg L^{-1}; Zn: 0.25 mg L^{-1}; Cu: 0.04 mg L^{-1}; and Mo: 0.03 mg L^{-1}.

At 6.5 weeks after sowing, nursery plants were transplanted to the open field on 25 April 2017. Before transplanting, potassium chloride and ammonium sulfate, stabilized with the nitrification inhibitor DCD (dicyandiamide) were applied by band placement at a soil depth of 20 cm and width of 30 cm along the center of the plots with a dosage of 47.6 g m^{-2} (ammonium sulfate), 0.48 g m^{-2} (DCD), and 50.0 g m^{-2} (KCL). Additionally, the band placement included three levels of triple superphosphate (TSP) application at 0, 1.25, and 5 g P m^{-2}, corresponding to 0, 12.5, and 50.0 kg P ha^{-1}, respectively. After transplanting, irrigation/fertigation in the field was performed by a dripper system with one lateral tube per row and drippers 25 cm apart. The distance between rows was 2 m and the distance between plants in the row was 25 cm. During field cultivation, additional fertigation without P was

employed to deliver nutrients to the plant roots into the wetted soil. The concentrations of N, K, Ca, and Mg in the fertigation solution were 80 mg L^{-1} (30% of N-NH_4), 75 mg L^{-1}, 200 mg L^{-1}, and 24 mg L^{-1}, respectively. Micronutrients were supplied at concentrations of 1 mg L^{-1} Fe, 0.5 mg L^{-1} Mn, 0.25 mg L^{-1} Zn, 0.04 mg L^{-1} Cu, and 0.03 mg L^{-1} Mo (for details see Supplementary Table S2). Irrigation was performed once a day and the amount was determined by the potential evaporation multiplied by the crop coefficient for each stage of plant development.

2.2.2. Microbial Inoculation

Inoculation was performed with two single-strain inoculants also used case study I (Proradix, FZB42), a combination product (Combifector B = CFB) based on *Trichoderma harzianum OMG16* and *Bacillus amyloliquefaciens FZB42*, enriched with Zn and Mn (Hochschule Anhalt, Bernburg, Germany, Abitep GmbH, Berlin, Germany) and the consortium product MCP (EuroChem Agro, Mannheim, Germany). The dosages of the inoculants are as follows.

Proradix WP suspension: 0.02% w/w, applied at a rate of 20 mL plant^{-1} in the nursery phase and 250 mL plant^{-1} applied after field transplanting.

Rhizovital 42 liquid formulation: 0.04% w/w, applied at a rate of 20 mL plant^{-1} in the nursery phase and 250 mL plant^{-1} applied after field transplanting.

CFB: in the nursery, each plant was supplied with 1 mL of a 1% (w/w) CFB suspension. At transplanting, each plant received 2 mL of a 2% (w/w) suspension

MCP suspension: 0.03% w/w—250 mL applied after field transplanting.

2.2.3. Plant Protection

No measures of plant protection were employed during the nursery phase. During open field culture, a range of different insecticides was repeatedly applied by canopy spraying during the culture period (Alaunt, Defender, Denim-Fit, Exirel, Floramite, Metronom, Mospilan, Oberon, and Pirate), as well as Vertimec by soil application. The major target was plant protection against various insects, especially mites and the tobacco white fly (*Bemisia tabaci*), as a vector of Tomato yellow leaf curl virus (for details see Supplementary Table S4).

2.2.4. Experimental Design and Statistical Evaluation

The experiment was arranged in a full factorial design (15 treatments with 5 BS variants × 3 P levels) in four randomized blocks. Each block included 15 plots. The length of each plot was 5 m and the distance between the centers of two adjacent plots was 2.0 m. Planting density was adjusted to 4 plants m^{-1} (2 plants m^{-2}).

Statistical analyses were performed by two-way ANOVA (treatments and blocks) with a Tukey test $p \leq 0.05$ for significance testing of treatment differences with JMP12.0 software package of SAS.

Additional statistical evaluations were performed also by three-way ANOVA (P dose, BS, and blocks) with a Tukey test $p \leq 0.05$ for significance testing of the overall major differences between the P dose treatments and the BS using the JMP13.0 software package of SAS (Supplementary Table S6).

2.2.5. Pre- and Postharvest Analysis

The experiment was terminated five months after sowing on 3 August 2017. Red fruits (approximately 80% red color) were selectively harvested on 20 July 2017 and all remaining fruits were harvested at the termination of the experiment. One representative plant was sampled from each plot on 20 July 2017. The following variables were measured; vegetative shoot (stem with leaves) biomass, root biomass and length, fruit yield (red, green, small fruits), and shoot P concentrations and content. The whole canopy including stems and leaves was removed aboveground and the rooted soil samples were collected in a diameter of 25 cm around the plant and a soil depth of 30 cm.

The roots were separated from the soil by washing with water over sieve. Separated roots segments were digitalized by scanning and root length was determined using the WinRhizo root

analysis software (Regent Instruments Inc., Quebec, QC, Canada). For determination of the P nutritional status, subsamples of the shoot tissue were oven-dried at 60 °C for three days, until the dry weight was constant, ground, and digested with concentrated sulfuric acid. The P concentration was determined with an automated photometric analyzer (Gallery plus, Thermo Fisher Scientific, Vantaa, Finland).

2.2.6. Soil Microbiome Amplicon Sequencing

DNA was extracted using the GenALL DNA extraction kit (GeneAll Biotechnology Co. Ltd., Seoul, South Korea) from root surface washings of rooted soil samples (rhizoplane, 200 mg; see Section 2.2.5) and from soil samples collected between the plant rows (300 mg), which still contained some roots. Therefore, this soil fraction was termed as "root-affected soil". The DNA was amplified with the primer pair CS1_515F (ACACTGACGACATGGTTCTACAGTGCCAGCMGCCG CGGTAA) and CS2_806R (TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT), and sequence libraries were generated. An Illumina MiSeq run was performed at the University of Illinois at Chicago Sequencing Core (UICSQC). This process yielded 22 Gb of information, and overall 3511942 sequences. These sequence data have been submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) databases under the BioProject PRJNA491280. Sequencing analysis was performed as follows; the sequences were paired, quality filtered, and chimeric sequences were removed by use of the 'mothur' software package [24]. Thereafter, the resulting sequences were clustered to operation taxonomic units (OTUs) based on 97% similarity (Table S7). Alpha and Beta diversity were calculated and the taxonomic affiliation was assigned with the QIIME software package [25], based on SILVA 123 database (https://www.arb-silva.de/download/archive/qiime/). Statistical analysis was performed in JMP Pro 13 (Statistical Analysis Software, SAS, Cary, NC, USA). The OTU rarefaction curve of soil and roots samples were computed using Vegan package in R.

The alpha diversity of bacterial communities indicated by the Shannon Diversity Index was determined in the root-affected soil collected between the plant rows and from root washings of the rhizoplane. Comparisons included the treatments with MCP versus single inoculants in the unfertilized control and in the variants with moderate P fertilization (12.5 kg P ha^{-1} soil). Beta diversity for soil and roots microbial community was estimated by nonmetric multidimensional scaling (nMDS) used to visualize the distances between the bacterial communities as calculated for the Bray–Curtis distance matrices.

3. Results

3.1. Case Study 1: Greenhouse Tomato Production in Timisoara, Romania 2016 and 2017

3.1.1. Growth of Nursery Plants

The tomato experiments carried out in 2016 and 2017 in Timisoara, Romania, revealed remarkable benefits of microbial BS applications already during nursery cultivation on the standard substrate mix used in the culture system. In both years, plant height and leaf area, determined as nondestructive indicators of plant performance at 43 days after sowing, were significantly increased by 29 to 100% (leaf area) and 29 to 74% (plant height) in response to BS application at the two leaves stage (Figure 1). However, in the two-year experimental period, neither the application of the *Bacillus* strain combination or the MCP treatment was associated with any consistent additional plant growth-promoting effect, as compared with the single-strain products.

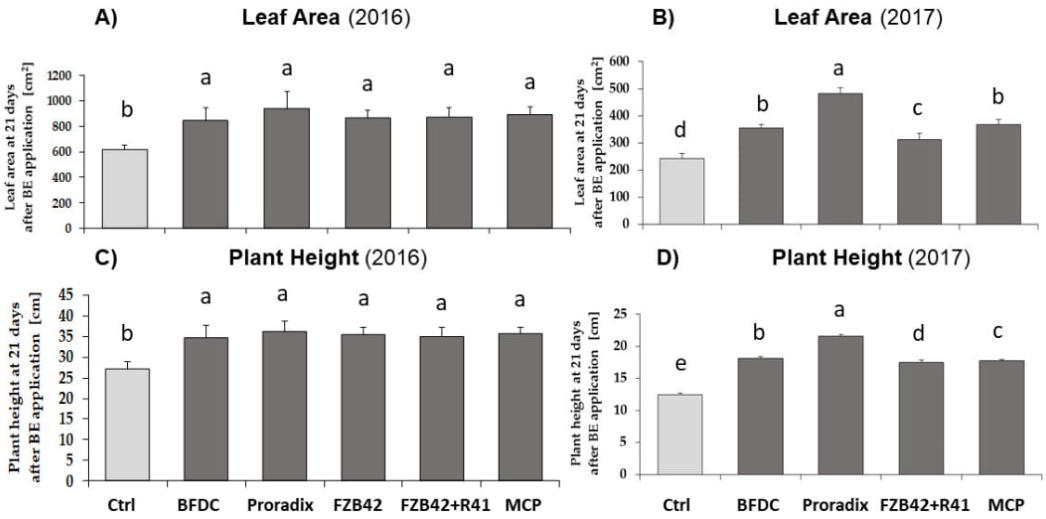

Figure 1. Leaf area (**A**,**B**) and plant height (**C**,**D**) of tomato cv Primadona F1 during the nursery phase at 43 days after sowing. Columns represent means ± standard deviation (n = 4 with each 10 plants as subsamples). Significant treatment differences (Tukey test, $p \leq 0.05$) are indicated by different characters.

3.1.2. Cumulative Fruit Yield

With approximately 70 t ha^{-1}, the control treatment did not reach the yield potential for greenhouse tomato production of 90 to 140 t ha^{-1} supplied with organic fertilizers [26]. By contrast, in both years, the cumulative yield of BS-treated plants was significantly increased by 39 to 84%, as compared with the untreated controls (Figure 2) with a yield range between 95 and 130 t ha^{-1}, which is in-line with the yield expectations. However, as compared with single strain inoculants, no additional yield improving effect was achieved by application of the *Bacillus* strains combination or the consortium product, and in 2016, even a significantly lower yield was recorded for the MCP treatment (Figure 2A).

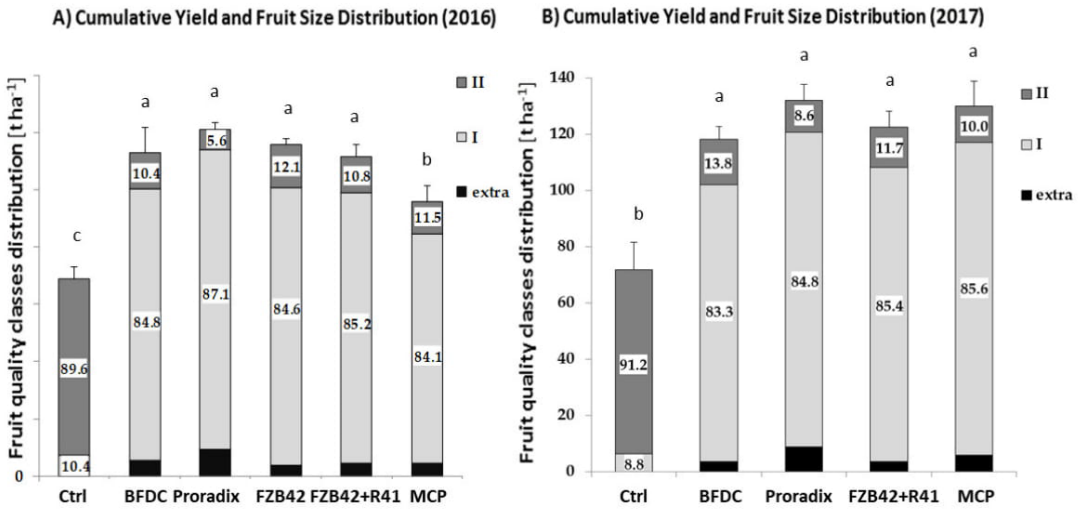

Figure 2. Cumulative yield (t ha^{-1}) and fruit size distribution (t ha^{-1} and %) for greenhouse tomato production with different BS treatments in 2016 (**A**) and 2017 (**B**). Quality classes (g FW fruit^{-1}): extra-large: >200 g, class I: 150–200 g, and class II: <150 g. Columns represent mean values of cumulative fruit yield ± SD (n = 4). Significant treatment differences in cumulative yield (Tukey test, $p \leq 0.05$) are indicated by different characters.

3.1.3. Distribution of Fruit Size and Seasonal Yield

In the control treatments, mainly class II fruits with a fresh biomass of less than 150 g were produced (approximately 90%) in both vegetation periods (Figure 2). By contrast, class I fruits (150–200 g) represented the dominant fruit size fraction (84–87%) in the BS-treated plants. The production of extra-large fruits (<200 g) was an exclusive feature of BS-treated plants (Figure 2). However, again no superior performance was detected for plants treated with MCP or the *Bacillus* strains combination as compared with the single-strain inoculants.

Regarding the seasonal yield distribution, BS inoculation promoted fruit ripening. This was reflected by 100 to 200% higher yield share during the main harvesting and marketing period in July as compared with untreated controls. This effect was similar for single strain and multiple inoculants as well (Figure 3).

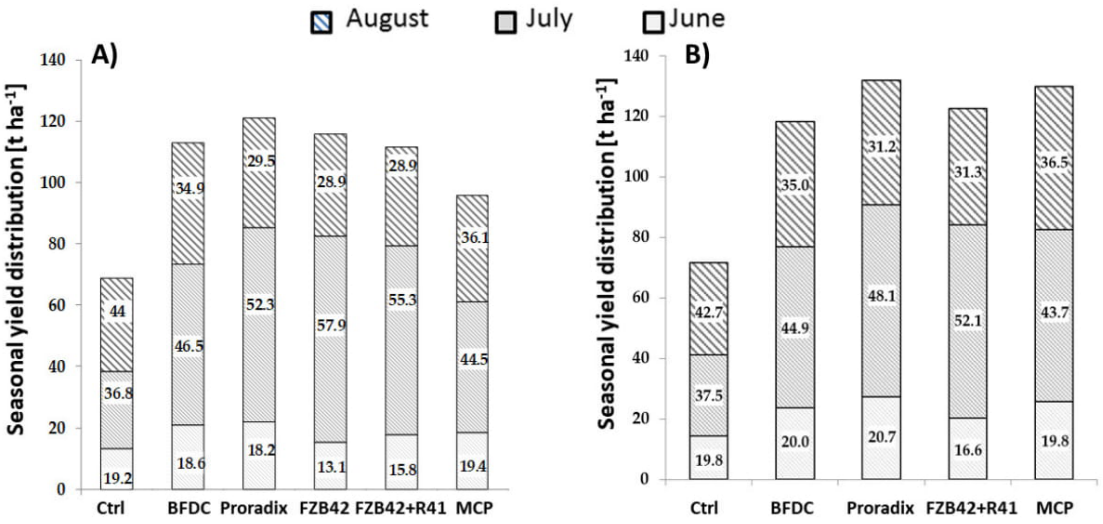

Figure 3. Seasonal yield share of tomato production over the whole harvesting period (June, July, and August) for different BS applications in Romania 2016 (**A**) and 2017 (**B**) (fruit yield in t ha^{-1}).

3.2. Case Study 2: Open Field Tomato Production with Drip Fertigation and Fertilizer Placement, Ramat Negev Desert, Israel, 2017

As expected, on the soil with alkaline pH 7.9 with low P availability (P_{Olsen} 5 mg kg^{-1} DM), P was the major limiting plant nutrient, indicated by continuously increasing shoot P concentration, plant biomass production and fruit yield with increasing levels of soluble TSP fertilization.

3.2.1. Vegetative Growth and Phosphate Status

In contrast to the greenhouse experiment in case study 1, only the *Bacillus–Trichoderma* combination product with Zn/Mn supplementation (CFB) and MCP treatments but not the single-strain inoculants exerted significant effects on early plant growth. CFB significantly increased shoot biomass production (24%) during vegetative growth (16 weeks after sowing) only in the variant with maximum P fertilization (50 kg P ha^{-1}), while exclusively, MCP significantly increased shoot biomass (113%) of the unfertilized control (Table 1). The effect of these products was not associated with corresponding changes in root biomass or root length. However, under maximum P supply, MCP significantly increased root length by 80% in comparison to the control in the investigated subsample without producing any effects on shoot growth (Table 1, Figure 4).

Table 1. Effect of banded P fertilization with DCD-stabilized ammonium sulfate and BS on the aboveground vegetative biomass production, root growth and shoot P status of tomato plants at 4 months after sowing, Ramat Negev, Israel. Data present means of four replicates. Statistical evaluation performed by two-way ANOVA. In each column, significant treatment differences (Tukey test, $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, are indicated by different characters, n.s. = not significant, * = significant after Tukey–Kramer Honest Significant Difference (HSD) test).

P Dose	Biostimulant	Shoot	Root	Root	Shoot P	Shoot P
		Biomass		Length	Concentration	Content
kg ha^{-1}		g plant^{-1}	g plant^{-1}	m plant^{-1}	mg g^{-1}	mg plant^{-1}
0	Control	300e	50.5	54ab	0.51g	23e
0	Proradix	340e	57.0	55ab	0.61fg	31de
0	FZB42	350de	62.8	63ab	0.67efg	36cde
0	CFB	260e	62.0	71ab	0.70efg	27de
0	MCP	640bc	36.7	46b	0.72efg	69abcde
12.5	Control	420bcde	51.2	47ab	0.78defg	49cde
12.5	Proradix	630bcd	42.2	42b	0.83def	78abcde
12.5	FZB42	400cde	46.3	58ab	0.87cdef	53bcde
12.5	CFB	430bcde	65.7	59ab	0.93cde	59bcde
12.5	MCP	500bcde	78.1	60ab	0.97bcde	73abcde
50	Control	620bcd	44.5	45b	1.01bcde	103abcde
50	Proradix	670bc	62.4	62ab	1.07bcd	106abcd
50	FZB42	680ab	58.6	70ab	1.16bc	119abc
50	CFB	770a	43.3	43b	1.28b	148a
50	MCP	500bcde	67.0	81a	1.87a	139ab
		Analysis of Variance				
	df	Shoot	Root	Root Length	Shoot P	
		Fresh weight			concentration	content
Treatment	14	**	ns	*	***	***
block	3	ns	ns	ns	ns	ns

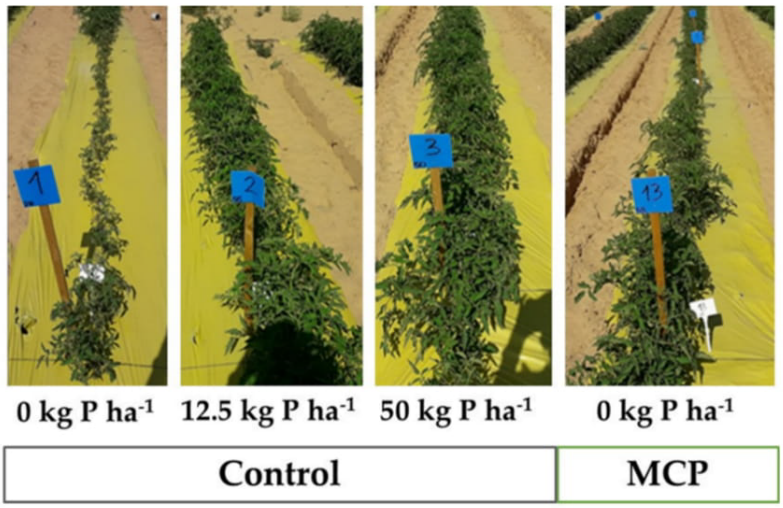

Figure 4. Effects of microbial consortia product (MCP) inoculation without external P fertilization on field performance of tomato plants at four months after sowing in comparison with different levels of P (triple superphosphate) fertilization in a field experiment at Ramat, Negev, Israel.

With a P shoot concentration of 0.05%, the control plants without P supply suffered from severe P limitation [27]. Accordingly, the application of TSP fertilizer increased the P nutritional status of the plants with a significant effect of 98% on P shoot concentration at the highest fertilization level in comparison with the unfertilized control, although the P tissue concentration was still suboptimal. A trend for a further improvement of the shoot P status was recorded for all BS treatments at all levels of P supply. However, a significant increase of 85% was obtained only for shoot P concentration of

the MCP treatment over the respective control when combined with the highest P dose of 50 kg ha^{-1} (Table 1). Analyzing the main effects of P dose and BS treatments revealed that both factors had a significant effect on shoot P ($F < 0.0001$ in both cases). The Tukey–Kramer separation test showed that MCP treatments were significantly different from all other BS treatments and CFB was significantly different as compared with the controls over all P doses (Table 1).

3.2.2. Fruit Yield

According to the improved P status, total fruit biomass significantly increased by 113% with a P supply of 12.5 kg ha^{-1} and by 232% with 50 kg P ha^{-1}, as compared with the unfertilized control (Table 2). The recorded BS effects on vegetative plant growth (Table 1) translated into a significant increase in final fruit biomass yield by 108% compared to the control only in the MCP variant without additional P fertilization, while no significant yield increase was recorded for the remaining inoculants. Similar trends were recorded for biomass and number of red fruits, although in this case the MCP effects were not significant. After supply of 12.5 kg P ha^{-1}, the yield effect of MCP was no longer significant compared with the untreated control and completely disappeared at the highest P fertilization level of 50 kg P ha^{-1} (see Table 2).

Table 2. Effect of banded P fertilization with DCD-stabilized ammonium sulfate and BS on total yield, fruit number per plant (No), and fruit quality distribution of drip-irrigated tomato between 4 and 5 months after sowing, Ramat Negev, Israel. Means represent four replicates. Statistical evaluation performed by two-way ANOVA. Significant treatment differences (Tukey test, $p \leq 0.05$ and Tukey–Kramer HD test) are indicated by different characters, n.s.: not significant, * = significant after Tukey–Kramer HD test).

P Dose	Bio Stimulant	Red Fruits		Green Fruits		Small Fruits		Total Yield	
kg ha^{-1}		t ha^{-1}	No	t ha^{-1}	No	t ha^{-1}	No	t ha^{-1}	No
0	Control	14.4e	13.5d	1.00	1.00	1.76	4.2	17.2b	187d
0	Proradix	21.7cde	19.8bcd	0.89	0.91	0.70	1.6	23.3ab	223bcd
0	FZB42	25.9 bcde	23.9abcd	0.69	0.69	0.72	1.6	27.3ab	262abcd
0	CFB	15.2de	16.1cd	0.74	0.72	1.53	3.7	17.5b	205cd
0	MCP	33.2bcde	27.5abcd	1.15	1.13	1.44	2.9	35.8a	315abcd
12.5	Control	36.1bcde	30.3abcd	0.30	0.25	0.34	0.7	36.7a	312abcd
12.5	Proradix	40.7bcd	27.8abcd	0.38	0.34	0.44	1.1	41.5a	293abcd
12.5	FZB42	33.1abcde	30.8abcd	0.00	0.00	0.00	0.0	33.1a	308abcd
12.5	CFB	31.9abcde	27.7abcd	0.29	0.31	0.57	1.6	31.7a	296abcd
12.5	MCP	45.5bc	32.4abc	0.29	0.34	0.49	1.3	46.3a	341abcd
50	Control	56.7a	40.6a	0.00	0.00	0.42	0.9	57.1a	415a
50	Proradix	57.0a	40.7a	0.35	0.44	0.43	1.1	57.8a	422a
50	FZB42	47.3abc	37.0ab	0.07	0.13	0.05	0.2	47.4a	374ab
50	CFB	52.9a	34.9ab	0.12	0.09	0.02	0.1	53.0a	350abc
50	MCP	50.8ab	36.1ab	0.08	0.09	0.13	0.6	51.1a	368ab
		Analysis of Variance							
	df	Red fruits		Green fruits		Small fruits		Total yield	
		Fresh weight	No	Fresh weight	No	Fresh weight	No	Fresh weight	No
Treatment	14	*	*	ns	ns	*	*	*	*
block	3	ns	ns	*	*	*	*	ns	ns

3.2.3. Microbiome Interactions

In face of the selective effects induced by the MCP treatments with respect to promotion of plant growth and yield formation, in case study II an amplicon sequencing approach was included to identify putative interactions of the BS with the soil microbiome, potentially related to the specific MCP effects.

Sequencing depth was adequate and as expected from highly complex environment root and soil bacterial communities (Figure S2). Although significant differences were found for the alpha diversity of root or soil samples treated with BS, no significant differences were found between the

examined treatments (two-way PERMANOVA nonparametric test) when the beta diversity of soil or root communities was analyzed using nonmetric multidimensional scaling (nMDS) calculated for the Bray–Curtis distance matrices (Figure S3).

In all treatments, the bacterial alpha diversity was lower at the rhizoplane as compared with the root-affected soil (Figure 5). In the control plants without BS inoculation, a significant decline in alpha diversity was recorded for the variant without P fertilization in comparison with the plants supplied with 12.5 kg P h^{-1}. This P fertilization effect on alpha diversity was not detectable in presence of the BS inoculants (Figure 5B). In the treatments without P supply, BS inoculation increased bacterial alpha diversity at the rhizoplane compared with the non-inoculated control, without significant differences between the different inoculants (Figure 5B). The BS inoculation effect on alpha diversity was similar to the effect of P fertilization. Moreover, the MCP inoculant significantly increased the alpha diversity also at the rhizoplane of the plants with P fertilization (Figure 5B). However, even in the root-affected soil samples, BS inoculation increased the bacterial alpha diversity with significant effects for FZB42 in the unfertilized control and for Proradix in the variant fertilized with 12.5 kg P ha^{-1} (Figure 5A).

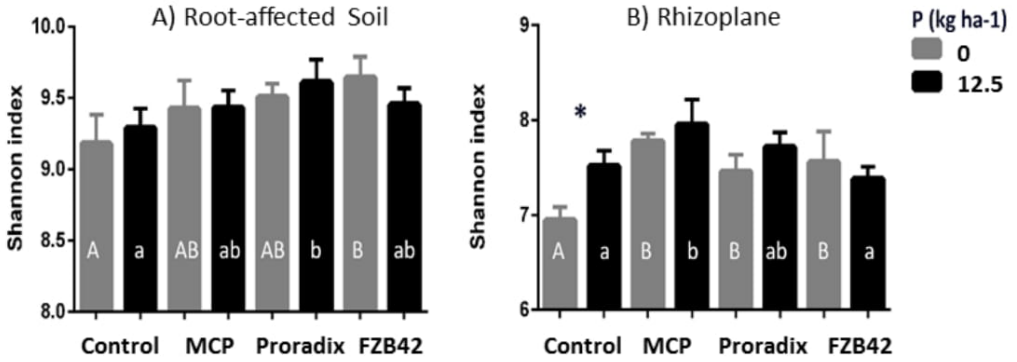

Figure 5. Shannon index for mean α-diversity of the bacterial communities in root-affected soil (**A**) and the rhizoplane (**B**) of drip-irrigated tomato plants with and without band placement of triple superphosphate (12.5 kg P ha^{-1}) and inoculation with different microbial biostimulants at 6 months after sowing, Negev Ramat, Israel. Significant differences (paired Student's *t*-test) in Shannon index between 0 and 12.5 kg P ha^{-1} dose of the same inoculant treatment are marked by *. Significant differences after pairwise comparison between inoculation treatments with the same P dose are indicated by different characters: A, B for 0 P, and a,b for 12.5 kg P ha^{-1}.

At the taxonomy level of class, *Acidobacteria*, *Nitrospira*, *Thermoleophilia*, and *Gemmatimonadetes* were detected exclusively in the root-affected soil but not at the rhizoplane, while *Flavobacteria* were detectable at the rhizoplane only. *Alphaproteobacteria* were dominant, both in the root-affected soil and in the rhizoplane–microbial communities. The abundance of *Actinobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Sphingobacteriia* was higher at the rhizoplane as compared with the root-affected soil samples in noninoculated control plants, while *Bacilli* and *Deltaproteobacteria* declined (Figure 6A,B). *Bacilli, Alpha-*, *Beta-*, and *Gammaproteobacteria* increased at the rhizoplane of P-deficient plants but the abundance of *Actinobacteria* and *Deltaproteobacteria* declined (Figure 6A,B). The inoculation with biostimulants was associated with a decrease in the abundance of *Sphingobacteriia* at the rhizoplane, and this effect was particularly expressed in P-deficient plants with MCP treatment (Figure 6B), associated with plant growth-promoting and yield-increasing effects. By contrast, the abundance of *Flavobacteria* was particularly high in the respective treatment (Figure 6B).

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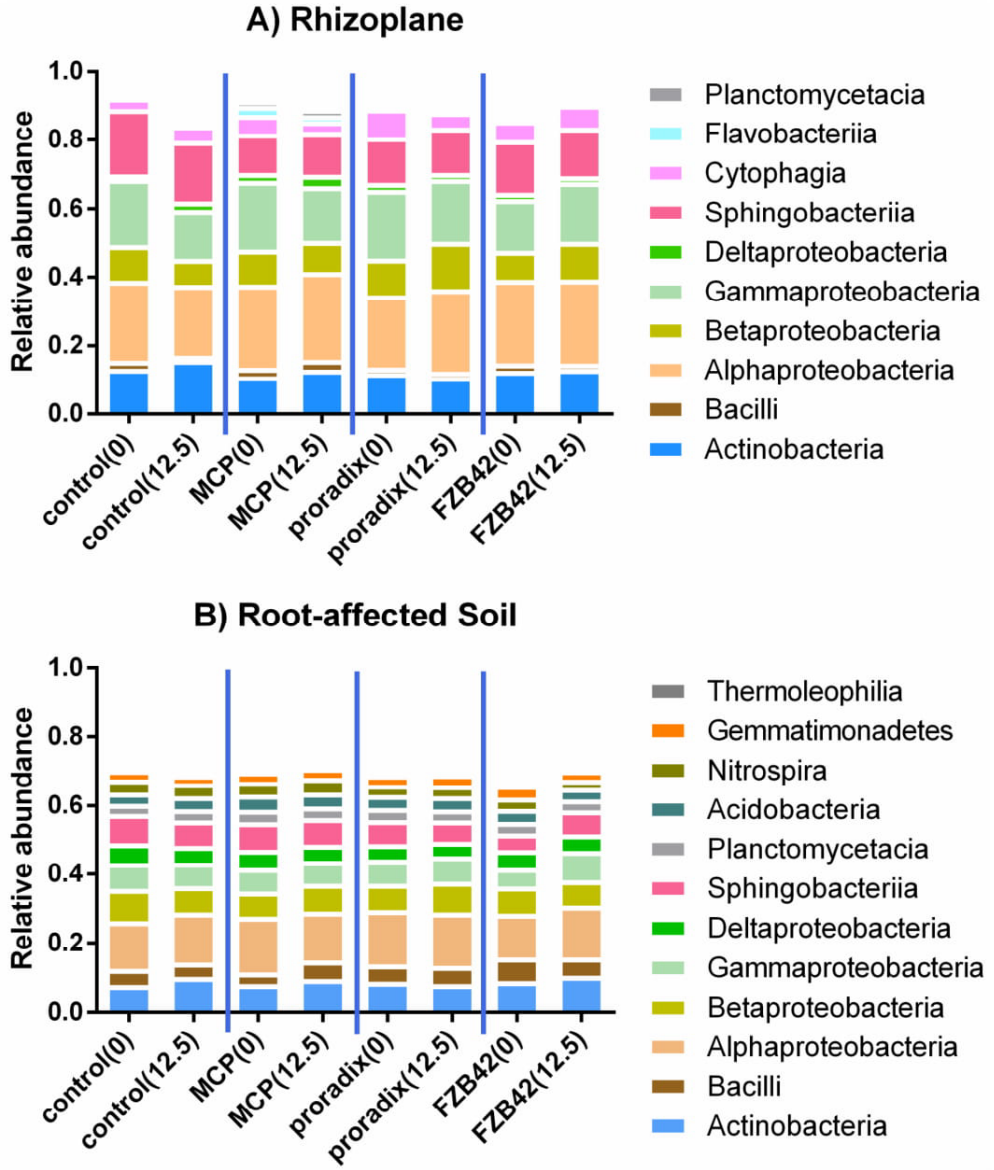

Figure 6. Relative abundance of different bacterial taxa at the rhizoplane (**A**) and in the root-affected soil (**B**) of drip-irrigated tomato plants with and without band placement of triple superphosphate (12.5 kg P ha^{-1}) and inoculation with different microbial BS at 6 months after sowing, Negev, Ramat, Israel.

4. Discussion

4.1. Case Study I: Large-Scale Greenhouse Experiments Timisoara, Romania, 2016/2017

In the large-scale greenhouse tomato production system in Romania, reproducible positive effects on the establishment of nursery plants, cumulative yield, fruit size distribution, and seasonal yield share were recorded in two successive vegetation periods.

4.1.1. Nursery and Vegetative Growth

In face of high nutrient contents of the organic nursery substrate (Supplementary Table S1), based on 45% composted cow manure amended with peat, soil and sand, the strong expression of BS-induced growth effects on nursery plants (Figure 1) was unexpected. However, in a comparative study on peat-based tomato nursery substrates, reduced plant biomass production and nutrient uptake was

associated with the application of manure fertilizers, frequently used in organic tomato production [28]. Maturation usually reduces the risk of phytotoxic effects of fresh manures and manure composts, while limitations in the availability of certain nutrients, such as Fe, Zn, and N, have been reported for mature composts [29,30]. Although the reasons for the suboptimal performance of the nursery plants in our studies are not entirely clear, the mitigation effect of BS applications is obvious (Figure 1) and may therefore, offer a perspective for optimization of nursery substrates frequently used also used in organic tomato production. Accordingly, for many of the microbial inoculants used in this study, root growth promoting and P-solubilizing properties are well documented [21,22,31–34]. The same holds true for priming effects against various abiotic and abiotic stresses [20,21,35–37] with protective effects also against potential substrate toxicities.

Inoculation with BS was performed during the nursery phase and just after transplanting into greenhouse culture. It remains to be established, whether the improved nursery plant performance after BS application (Figure 1), finally translated into the observed beneficial yield effects (Figure 2). Alternatively, this may be attributed to more direct effects, induced by long-lasting BS colonization during maturation of the host plants. Tomato is a plant species with documented ability to release root secretory acid phosphatases under P limitation [38] with potential to hydrolyze organic P forms abundant in manure-based fertilizers. Moreover, increased P deficiency-induced root extrusion of protons [39,40] can contribute to solubilization of acid-soluble mineral soil P forms. Strengthening and root growth promotion of nursery plants after BS inoculation may therefore improve the utilization of the applied organic fertilizers. On the other hand, phosphatase secretion and mobilization of sparingly soluble mineral phosphates, mycorrhizal helper functions, as well as contributions to N turnover and N fixation, are features also documented for the microbial BS used in the present study [11,15,32,33,41]. Therefore, in case of longer lasting rhizosphere survival, also direct contributions of the inoculants to plant growth promotion and nutrient acquisition from the organic fertilizers in the production phase are a likely scenario, at least in the 2017 experiment. For phytosanitary reasons, in this case, plant culture was performed in substrate containers, with a rooting volume restricted to 10 L. The basal substrate fertilization was dominated by a mixed hair/feather meal fertilizer (Monterra 13% N, 0.22% P; 10 g L^{-1} substrate), supplemented with mineral N, P, and K at a rate of 140, 70, and 149 mg L^{-1} substrate, respectively. Thus a better exploitation of the available rooting volume by BS-induced root growth promotion and improved utilization of the organic fertilizers as previously reported in the literature [9,11,23,42,43], would represent an advantage under the respective growth conditions. The same holds true for P acquisition in face of the moderate P fertilization level and low background P availability of the unfertilized substrate (Supplementary Table S1).

Organic fertilization was dominant also in the 2016 experiment and applied as a mixed guano/feather meal product (DIX-10N, 10% N, 1.3% P, 2 t ha^{-1}). Nevertheless, limitations in nutrient availability of the substrate seem to be unlikely in this case, since nutrient analysis of the greenhouse soil revealed high background levels of plant-available P and N_{min} (Supplementary Table S1). However, as an important challenge, in the 2016 experiment, the tomato plants showed symptoms of root rot induced by the soil-borne pathogen *Fusarium oxysporum* Schlecht f. sp. *radicis-lycopersici* Jarvis and Shoemaker. Additionally, increased larvae abundance of *Agriotes lineatus* L., that can feed on the roots of tomato plants was recorded as well. In this context, biocontrol properties and the ability to induce systemic resistance or improved plant vitality and root growth, as reported for the inoculated BS [20,37,41,44], could represent an additional advantage. The BS inoculants may contribute to compensation of pathogen-induced root damage, thereby determining the observed effects on plant growth promotion and yield formation. Nevertheless, independent of pathogen suppression, in all three scenarios described in case study I, microbial root growth stimulation and nutrient mobilization as documented features of the applied inoculants, would definitely represent a beneficial factor, either supporting nutrient acquisition under limited nutrient availability (in 2017) or by counteracting inhibition of root growth and activity due to nursery substrate toxicities or pathogen infection.

4.1.2. Generative Growth and Fruit Yield

The application of BS increased the individual fruit weight by 20 to 30% whereas the total fruit biomass production per plant was promoted even more strongly by approximately 40 to 75% (Supplementary Table S5). This finding suggests that BS application particularly increased the number of fruits per plant and to a lesser extent the growth of individual fruits. This may be attributed to beneficial effects on flowering and fruit setting as processes under hormonal control [45]. Experiments with exogenous application of plant growth regulators and measurements of internal changes in hormone concentrations, suggest an important role of auxins in this context [45–47]. This raises the question whether the well-documented potential of the selected inoculants for auxin production [21,48] or their interactions with plant hormonal balance might be involved in the observed BS-induced promotion of fruit setting and fruit growth. In experiments testing different single-strain and mixed BS, similar effects on tomato growth and yield formation have been recently reported by Oancea et al. [49]. Microbial BS based on *Azospirillum lipoferum* and *Brevibacillus parabrevis* proved to increase total marketable tomato yield by more than 10%. The authors speculated that the effects were due to accelerated vegetative growth and quicker development during the early growth of tomato plants. The fruits had the chance to ripe more rapidly, which improved the commercial fruit quality and the weight of marketable fruits since the earlier ripening of fruits ensures better competitiveness for the farmers, as similarly observed also in the present study (Figure 3). Although numerous studies show beneficial effects of microbial BS particularly on flowering, fruit setting and fruit development of tomato and other fruit crops [49–52], the underlying modes of action still remain to be elucidated.

Single Strains versus Microbial Consortia

Interestingly, fungal and bacterial BS of different phylogenetic origin (strains of *Penicillium*, *Bacillus*, and *Pseudomonas)* as well as single-strain inoculants versus microbial consortia exhibited very similar stimulatory effects on plant growth and yield formation (Figures 1 and 2). There was no indication for an improved performance of strain combinations in comparison with single strains, previously postulated as an advantage of consortium products in various literature reviews. As a possible explanation for this observation, the stress-protected nursery in small pots with a small soil volume, followed by protected greenhouse culture, may offer optimal conditions for effective root colonization by the selected microbial BS, as a prerequisite for the establishment of efficient plant-inoculant interactions in the rhizosphere. Environmental stress factors, such as temperature or pH extremes, limited or excess water supply, oxygen limitation, salinity, etc., were largely excluded. Under these conditions, the beneficial effects of BS inoculation may be limited rather by the genetically fixed response potential of the host plants than by the plant growth-promoting properties of the inoculants. Therefore, obviously maximum growth and yield responses, reaching the reported yield potential for organic greenhouse tomato production [26], were already induced by the single strain inoculants leaving no further scope for additional effects of combination products.

4.2. Case Study II: Open Field Tomato prOduction with Drip Fertigation and Fertilizer Placement, Ramat Negev Desert, Israel, 2017

A completely different scenario was observed under the more extreme environmental conditions in case study II. Although, similar to the experiments in Romania, nursery culture, and BS inoculation were performed under protected conditions, subsequent open field culture in the Negev desert was of course more challenging for plant growth. High temperatures and radiation intensities (daytime temperature 30–42 °C, radiation: 1000 W m^{-2}), lack of precipitation throughout the whole culture period, high soil pH, low soil fertility, and organic matter content as well as limited P availability represented major challenges in this production system (Supplementary Figure S1, Table S1). This may be related with induction of multiple stresses including nutrient deficiencies, limited plant-beneficial soil microbial activities, heat stress, excessive transpiration, and oxidative stress due to high light intensities. Although water and nutrients were supplied by fertilizer drip

fertigation and fertilizer placement, a drip irrigation system may be associated with some limitations under these challenging environmental conditions due to rapid evaporation and concentration of nutrient salts in the application zone.

4.2.1. Vegetative Plant Growth and Yield Responses

In contrast to the greenhouse experiments in Romania, only combination products successfully induced plant growth stimulation, while single strain inoculants were largely ineffective (Table 1). Stimulation of yield formation was observed exclusively for the MCP treatments, particularly under conditions of P limitation (Table 2), identified as limiting nutrient. With increasing levels of P supply, the P nutritional status, plant growth, and fruit yield increased, while the MCP effect finally disappeared (Table 2). Although tomato is a plant species with documented potential to acidify the rhizosphere under P-limited conditions [39,40], this effect was obviously not sufficient to mobilize significant amounts of acid soluble P forms on the alkaline soil. Even a further promotion of the acidification effect by placement of a stabilized ammonium fertilizer, leading to localized root proliferation and ammonium-induced proton extrusion [53], was not effective in this context. Only the additional MCP inoculation increased vegetative plant growth and fruit yield of plants without external P supply to a level comparable with a moderate P fertilization level of 12.5 kg P ha^{-1} (Table 2). MCP inoculation of plants supplied with 12.5 kg P ha^{-1} even resulted in a yield increase not significantly different from the fully fertilized positive control with 50 kg P ha^{-1} (Table 2), reflected also by a similar P nutritional status in both treatments (Table 1). This finding points to a significant contribution of the MCP inoculants to P acquisition of the host plant. Although no BS-induced promotion of root length development with beneficial effects on spatial P acquisition was detectable (Table 2), local root growth stimulation close to the ammonium fertilizer depot, as recently described also by Nkebiwe et al. [22], cannot be excluded. In this context, it must be taken into consideration that the related locally restricted root growth modifications are not easily detected by excavation of whole root systems under field conditions. Moreover, the MCP inoculant provided a wide range of microbial genera (*Bacillus*, *Pseudomonas*, *Trichoderma*, *Penicillium*, and *Aspergillus*) with documented P-solubilizing properties [32,33,54]. Phosphate limitation is also associated with a rapid inhibition of N-uptake and -assimilation [35,36]. This effect may be particularly expressed in case of ammonium-dominated fertilization due to lower soil mobility of ammonium compared with nitrate. In this context, the presence of *Nitrobacter*, *Nitrosomonas*, and *Azotobacter* in the MCP inoculant may contribute to improved N availability by nitrification and N_2 fixation. Compared with single strain inoculants, the MCP product may therefore offer a larger flexibility, by providing a whole range of root growth-promoting and/or P-solubilizing strains, which may differ in their sensitivity to environmental stress factors. This will increase the probability for the expression of beneficial effects on crop performance, even under the more adverse environmental conditions in the selected culture system.

Interestingly also the *Bacillus*/*Trichoderma* combination product, amended with Zn and Mn (CFB), exerted some growth-promoting effects during vegetative plant development (Table 1). However, in contrast to the MCP product, these effects were restricted to the treatments with the highest mineral P fertilization (50 kg P ha^{-1}). On alkaline soils, limited micronutrient availability (e.g., Zn, Mn, and Fe) is frequently a growth-limiting factor. Particularly external and internal Zn availability can be further reduced by high levels of P fertilization [55,56] and Zinc limitation is associated with shoot growth depression and impairment of defense responses against oxidative stress [27]. Although, the P nutritional status of the plants supplied with 50 kg P ha^{-1} was not extraordinarily high (Table 1), the application mode via band placement implicates high local P concentrations. Therefore, a certain degree of Zn limitation may also represent a problem in the present study on the alkaline pH 7.9 soil in the treatments with the highest level of P supply, required to overcome low soil P availability, and mitigated by supplementation of Zn with CFB inoculation.

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4.2.2. Interactions with the Soil Microbiome

In face of the significant and highly selective plant growth-promoting and yield-increasing effects of MCP inoculation in case study II (Tables 1 and 2) we decided to characterize also interactions with the soil microbiome in comparison with the ineffective single-strain inoculants. The aim of these investigations was to identify potential indirect plant growth-promoting modes of action via changes in soil bacterial communities. An amplicon sequencing approach revealed a lower alpha diversity of bacterial communities at the rhizoplane as compared with the root-affected soil between the plant rows (Figure 5). Similar effects have been reported also in previous studies [57–59] and may reflect the selective impact of the root on microbial communities. Accordingly, plant-, and even cultivar-specific patterns in the composition of root exudates and rhizodeposits, as well as specific root-induced modifications of physicochemical rhizosphere properties have been described [60]. The rhizoplane alpha diversity was also lower under P limitation compared to variants with P fertilization (Figure 5). This may reflect specific adaptive modifications of the rhizosphere conditions by the host plant towards improved P acquisition, such as rhizosphere acidification, increased release of organic metal chelators and phenolic compounds, phosphatases, chitinases, etc. [61], with a selective impact on rhizosphere-microbial communities. Interestingly, inoculation of the microbial biostimulants increased the alpha diversity at the rhizoplane under P limitation, which was particularly expressed in the MCP treatments (Figure 5), and may be regarded as consequence of an improved plant P nutritional status in these variants (Table 2).

However, increased rhizoplane–microbial diversity may also increase the probability for the establishment of beneficial plant-microbial interactions and some apparent changes were detectable at the taxonomy level of class. Particularly in the MCP treatments, with the highest plant growth-promoting and yield-increasing potential, a distinctly increased abundance of *Sphingobacteriia* was recorded at the rhizoplane as compared with the root-affected soil (Figure 6). Sphingobacteriia are known as salinity indicators [62,63], and increased accumulation of salts in the rhizosphere is characteristic for plants exposed to high transpiration and/or drought [64], as stress factors affecting also the investigated tomato production system under desert conditions (Supplementary Figure S1). High water evaporation due to high temperatures, water uptake by the plants and the comparatively low water supply by drip irrigation are factors increasing the concentrations of minerals in the rhizosphere soil solution, and may promote the accumulation of salts, as indicated by a higher abundance of salinity-adapted Sphingobacteriia at the rhizoplane. Interestingly, this effect was at least partially reverted in response to microbial inoculation, particularly in the MCP variants (Figure 6A). For various PGPRs, arbuscular mycorrhizae, and also plant roots, the ability to increase aggregate stability and the water-holding capacity of the rhizosphere soil by secretion of exopolysaccharides and glomalin is well-documented [65–67]. The resulting higher rhizosphere hydration would consequently reduce the salt concentrations in the rhizosphere soil solution and may explain the lower abundance of *Sphingobacteriia* as salinity indicators. Moreover, higher water content in the rhizosphere would also improve the nutrient availability under drought stress conditions. Particularly for members of the genera *Pseudomonas* and *Bacillus* as dominant bacterial groups in the MCP inoculant, exopolysaccharide production with the potential to promote drought and salinity tolerance of host plants but also mycorrhizal helper functions have been identified [11,41,67,68]. These inoculants might therefore contribute to the superior plant growth-promoting potential of MCP under the investigated culture conditions. *Flavobacteria* represented another bacterial group, exclusively detectable at the rhizoplane particularly in MCP-inoculated plants without external P supply (Figure 6B). For *Flavobacteria*, PGPR properties [69–71] and a role as drought stress protectants [72] have been reported in the literature.

However, with the exception of bacilli in the P-fertilized control (Figure 6A), there was no indication for an increased abundance of bacterial groups that are reported to be present in the MCP inoculant, suggesting indirect effects of the BS products on the microbiome composition, rather than a direct introduction of the respective genera by BS inoculation. Moreover, the evident increase in alpha diversity at the rhizoplane of BS-treated plants suggests this indirect effect to be selective

for the root-associated microbiome, particularly under conditions of low P availability. However, the microbiome analysis was conducted approximately four months after inoculation and therefore direct interactions in the earlier growth stages with plant growth-promoting effects of a beneficial consortium (MCP) on the low fertility soil with limited microbial activity cannot be excluded. For a more accurate examination, inoculant tracing would be required during the culture period, which would be a particularly challenging task for consortium products, due to the large number of inoculated strains. However, fertigation-based culture systems may offer a suitable approach for comparing the effectiveness and economy of starter application versus repeated inoculations. Particularly with subsurface fertigation tubes, it should be possible to perform effective repeated inoculations of the rooting zone even in later stages of plant development. This could provide important information to the question whether BS treatments are more suitable to support the sensitive phase of crop establishment with indirect effects on later plant development or whether a longer lasting rhizosphere establishment would be more effective.

5. Conclusions

The results of the present study clearly indicate a plant growth-promoting and yield-increasing potential of various fungal and bacterial BS in tomato production. Although the modes of action are not entirely clear, the results suggest that direct plant growth-promoting activities providing improved start conditions already during early growth stages enabled the plants to utilize a given nutrient supply more effectively and increased the stress resistance, translating into tremendous yield increases particularly under conditions of suboptimal nutrient acquisition. Furthermore, stimulation of flowering or fruit setting and fruit size development, have been observed in response to the BS application during early growth, indicating long-lasting effects on plant development. The data also demonstrate that the performance of microbial consortia is not always superior over single-strain inoculants. In accordance with the concept of an improved adaptive potential postulated for MCPs, a clear advantage in comparison with single-strain inoculants was recorded in the drip-irrigated tomato production system in the Negev desert, exposed to various environmental challenges, such as high temperature, limitations in water availability, low soil fertility, and high soil pH. By contrast, superior MCP performance was not detectable under the more controlled and less challenging conditions in the greenhouse production system in Romania, where all inoculants showed similar plant growth-promoting effects. Since two different tomato cultivars, characteristic for the two different production systems, were used for the experiments, cultivar-specific responsiveness to BS inoculation cannot be completely excluded as an alternative explanation for the observed differences in the expression of BS effects. However, for the selected inoculants, a broad efficiency spectrum in combination with a wide range of different crops has been reported in the literature [20,21,23,31,73]. These findings do not suggest highly selective strain- and cultivar-specific plant–BS interactions at least for the investigated single strain inoculants.

The selective plant growth-promoting MCP effects in the drip-irrigated tomato production system with limited P supply were also associated with some characteristic modifications of rhizosphere–bacterial communities. MCP inoculation increased the bacterial alpha diversity at the rhizoplane of P-limited tomato plants. The abundance of *Sphingobacteriia*, known as salinity indicators, declined while the population of potentially plant growth-promoting and drought stress-protective *Flavobacteria* increased. Although the observed effects suggest some MCP-mediated interactions with the expression of stress-adaptive processes also related with alterations of the rhizosphere microbiome, it is still not clear whether these effects must be regarded as a cause or rather as a consequence of an improved stress adaptation of the MCP-inoculated tomato plants.

Nevertheless, the presented findings support the hypothesis that the use of microbial consortia can serve as a tool to increase the efficiency and reproducibility of BS-assisted strategies for crop production, particularly under challenging environmental conditions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/2/105/s1, Figure S1: Climate parameters (air temperature 0.5 m above ground, relative air humidity, and radiation) during the experiment in Ramat Hanegev, Israel; Figure S2: Sample rarefaction curves for soil (a) and root (b) samples; Figure S3: Nonparametric multidimensional scaling (nMDS) analysis of root-affected soil (a) and rhizoplane (b) microbiome; Table S1: Substrate properties; Table S2: Fertilization management; Table S3: Application of biostimulants; Table S4: Plant protection; Table S5: Fresh biomass of individual fruits and cumulative fruit biomass production per plant for greenhouse tomato production in Romania with different BS treatments in 2016; Table S6: Three-way ANOVA (P dose, biostimulants, and blocks) on effects of banded P fertilization with DCD-stabilized ammonium sulfate and biostimulants on vegetative shoot and root biomass, root length, P nutritional status, total yield, fruit number per plant (No), and fruit quality distribution of drip-irrigated tomato, Ramat Negev, Israel; Table S7: OTU tables for all soil/rhizoplane samples. In root affected soil, a total of 7815 OTUs were found: 7200 Bacterial, 252 Archaeal, and 363 unassigned. At the rhizoplane, there were 5218 OTUs overall: 4931 Bacterial, 37 Archaeal, and 250 unassigned. Sample names indicate the treatment (Control, MCP, Proradix, FZB421), phosphate level (0, 12.5), and replicate (Rep A–D).

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5.2 Impact of microbial biostimulants on utilization of poultry manure and drought stress tolerance of spring wheat

Original title: "Einfluss mikrobieller Bioeffektoren auf P-Aneignung und Trockenstresstoleranz bei Sommerweizen"

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Background and objectives

In face of repeated reports on preferential performance of various PGPM inoculants, including MCPs in combination with manure based organic fertilizers in greenhouse trials with maize and tomato (Thonar et al.2017; Mpanga et al., 2018; Vinci et al, 2018a, b; Bradáčová et al. 2019c), this study was initiated as a systematic comparison of single strain inoculants, microbial combination products and the MCP inoculant with spring wheat as a typical field crop. The field experiment was established on a clay loam soil with low mineral P availability (P_{CAL} 20 mg kg^{-1} soil) supplied with a standardized organic pellet fertilizer based on poultry manure and meat meal (MP, Agriges, San Salvatore Telesino. Italy).

Superphosphate (SP) and calcium-ammonium nitrate (CAN) variants were included for comparison as mineral fertilizers. Due to severe water limitation during the establishment phase in April, the experiment also offered the opportunity to evaluate potential drought-protective properties of the inoculants, as frequently reported in literature for various PGPMs (Schütz et al., 2017).

Hypotheses

- The microbial BS are able to improve growth of spring wheat under drought stress, during the sensitive establishment growth phase. This beneficial effect is finally translated into increased grain yield.
- The utilization of the manure-based MP fertilizer is improved by BS inoculation and stimulates plant growth and yield formation.

- MCP and combination products will show an advantage in terms of plant growth promotion and yield as compared with the single-strain inoculant.

Methodology

The field experiment was carried out on a clay-loam soil with low mineral P availability (Fluvisol of the Werra river valley, clay-loam, pH ($CaCl_2$) 5.9; 20 mg P (CAL) kg^{-1} soil) located at the research station Heßberg near Hildburghausen, Thüringen, Germany from 28.03. to 29.08.2017 (**Fig. 4**). Microbial inoculants based on the MCP (ECAG 2895, Eurochem Agro GmbH, Mannheim, Germany), and the combination product Combifector B (*Trichoderma harzianum* OMG16 + *Bacillus velezensis* FZB42 + Zn/Mn supplementation; Anhalt University of Applied Sciences, Bernburg, Germany) were investigated in comparison with the single strain inoculant *Bacillus subtilis* CH13 (ECAG 2920, Eurochem Agro GmbH, Mannheim, Germany) in combinations with a pelleted organic fertilizer (MP) based on poultry manure and meat meal (3.1% N, 1.3% P, 1.1% K (MP, Agriges, San Salvatore Telesino, Italy). The microbial BS products were applied by soil drenching, diluted in water (20 L $plot^{-1}$) before sowing according to the instructions of the manufacturers at a dosage of 375g ha^{-1} for CFB, 2.5 liter ha^{-1} for MCP and 27.8 liter ha^{-1} for *B. subtilis* CH13 According to the local farming practice, the standard (**Strd**) fertilization comprised two rates of N fertilization (70 kg N ha^{-1} as calcium ammonium nitrate (CAN) at seedling emergence and 35 kg N ha^{-1} as CAN at tillering). The tested fertilization strategies were: **Zero** = no fertilization; 46 kg P ha^{-1} as super phosphate (**SP**) before sowing, **MP** = 105 kg N + 46 kg P ha^{-1} as manure pellets (MP); and the positive control with fully adequate N and P fertilization (**Strd + SP** as described before). In addition, a basal fertilization with 35 kg P ha^{-1} as rock phosphate (P40; 17 % P) in autumn 2016 and 20 kg S + 15 kg Mg ha^{-1} as Kieserite at stem elongation was applied to all variants.

Sowing of spring wheat (Triticum *aestivum* L. cv. Alora, Baywa, Römhild, Germany) was performed at 29.03.2017 with a sowing density of 400 seeds m^{-2} and 20 rows $plot^{-1}$ (plot size 32 m^2) in a row distance of 13 cm. Plant protection was performed by initial fungicide seed dressing with Landor® CT, based on Fludioxonil, Difenoconazol and Tebuconazol. During the culture period, a herbicide treatment (Biathlon 70 g ha^{-1}) was performed on 06.05.2017 at 38 days after sowing (DAS) followed by application of the growth regulator Moddus (0.2 L ha^{-1}) on 02.06.2017 (65 DAS) and a fungicide/insecticide treatment (Aviator-Pro 1.2 L ha^{-1} + Karate-Zeon 75 mL ha^{-1}) on 15.06.2017 at 79 DAS. Final harvest was performed on 08,09,2017.

Fig. 4: Field site in Heßberg, Thüringen (Neundorf, 2017)

Results and discussion

In 2017, the establishment phase of wheat was strongly affected by early spring drought with very low precipitation especially at the begin of the vegetation period directly after sowing by the end of March (**Fig. 5**).

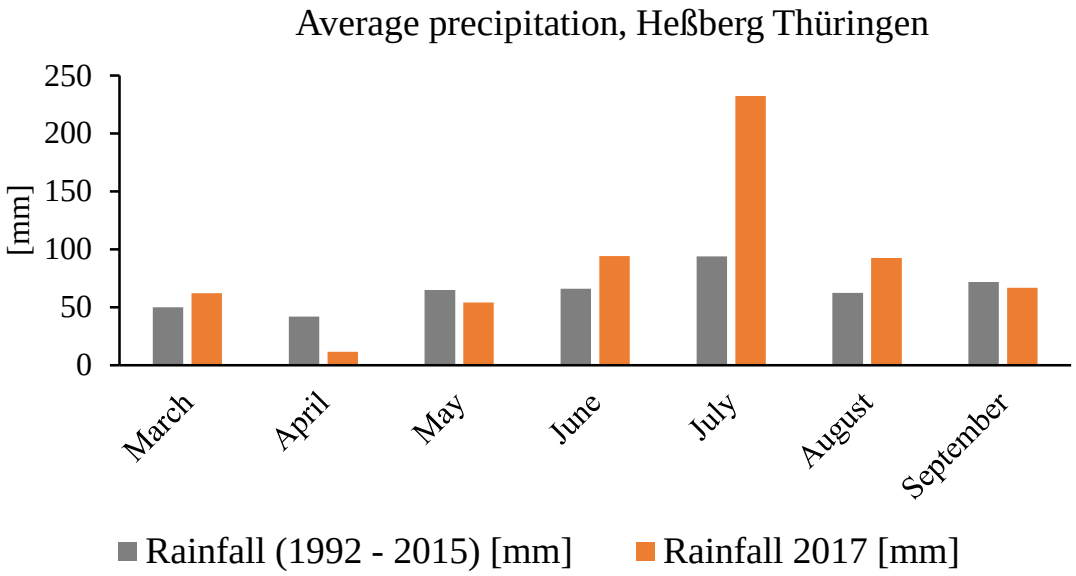

Fig. 5: Average precipitation during the vegetation period of the spring wheat experiment Heßberg, Thüringen, 2017 in comparison with a long-term rainfall statistics (1992-2015) in Thüringen, Germany (adopted from wetter-th.de on 18.02. 2018).

This was associated with a delayed and weak seedling establishment and poor tillering, with only 1.9 tillers plant^{-1} on average at 58 DAS and a low number of ear-bearing tillers, (**Fig. 7**). Although no additional stress events were recorded during the further culture period,

the average grain yield of spring wheat in this experiment (around 4 t ha^{-1}) was very low in comparison with average yield of spring wheat recorded in Thüringen, Germany in 2017 (6.9 t ha^{-1}) (TLL, 2018).

Aerial photographs (**Fig. 6**) as well as nutrient nalysis of the flag leaves at 72 DAS (**Fig. 8**) indicated that rather N than P was a limiting factor, despite low available P concentrations in the soil. Apart from heterogeneity of the field site, the aerial photograph shows a clear greening effect of the CAN and also the organic MP treatment but no effect of soluble SP application, suggesting a supplementation mainly of N rather than P limitation, both by the mineral and organic fertilizers. This was confirmed by determination of the plant nutrirional status via flag leaf analysis at 74 DAS, showing N limitation but no P limitation in all variants without inorganic (CAN) or organic (MP) N supply (**Fig. 8**), although the available mineral soil P status of 20 mg P_{CAL} kg^{-1} was low (VDLUFA, 2018). This may be explained by high organic soil P reserves, since the field site was recently established from grassland conversion. This is also reflected by a high organic matter content (2.8 %) and high activities of enzymes involved inorganic, C, N and P cycling (Bradáčová et al., 2019a, b). Accordingly, only the application of mineral or organic N fertilizers was associated with a significant increase in final grain yield in comparison with soluble P (SP) supply or without fertilization (**Fig. 7**) Interestingly, also the plant K status was critical in all treatments while the status of Mg Ca, Zn and Mn reached the sufficiency range.

Fig. 6: Re-compilation of an aerial drone photograph of the experimental site in Heßberg, Thüringen taken at 102 DAS, showing the different treatment blocks. 0 Ctrl = unfertilized control; SP = Superphosphate; CAN = calcium-ammonium nitrate; SP + CAN = Superphosphate + calcium-ammonium-nitrate; MCP = unfertilized control + MCP; MP = poultry manure pellets; CFB = Combifector-B; CH13 = Bacillus subtilis CH13. Perzelle fehlerhaft = fertilization error (Neundorf, 2017).

No additional effects on seedling emergence, early growth, nutritional status or final grain yield were recorded for the BS treatments. This may be attributed to the drought stress phase during rhizosphere establishment of the inoculants, associated with weak seedling development. As repeatedly demonstrated in earlier studies, plants affected by external stress factors are frequently not able to support the development of a successful interaction with PGPMs in the rhizosphere (Bittman et al., 2006; Lekfeld et al., 2016; Thonar et al., 2017; Mpanga et al., 2019). Moreover, potential incompatibilities of the inoculants with the applied plant prodection agents may offer an additional explanation. In terms of P acquisition, BS effects could not be expected since the P nutritional status was sufficient (**Fig. 8**).

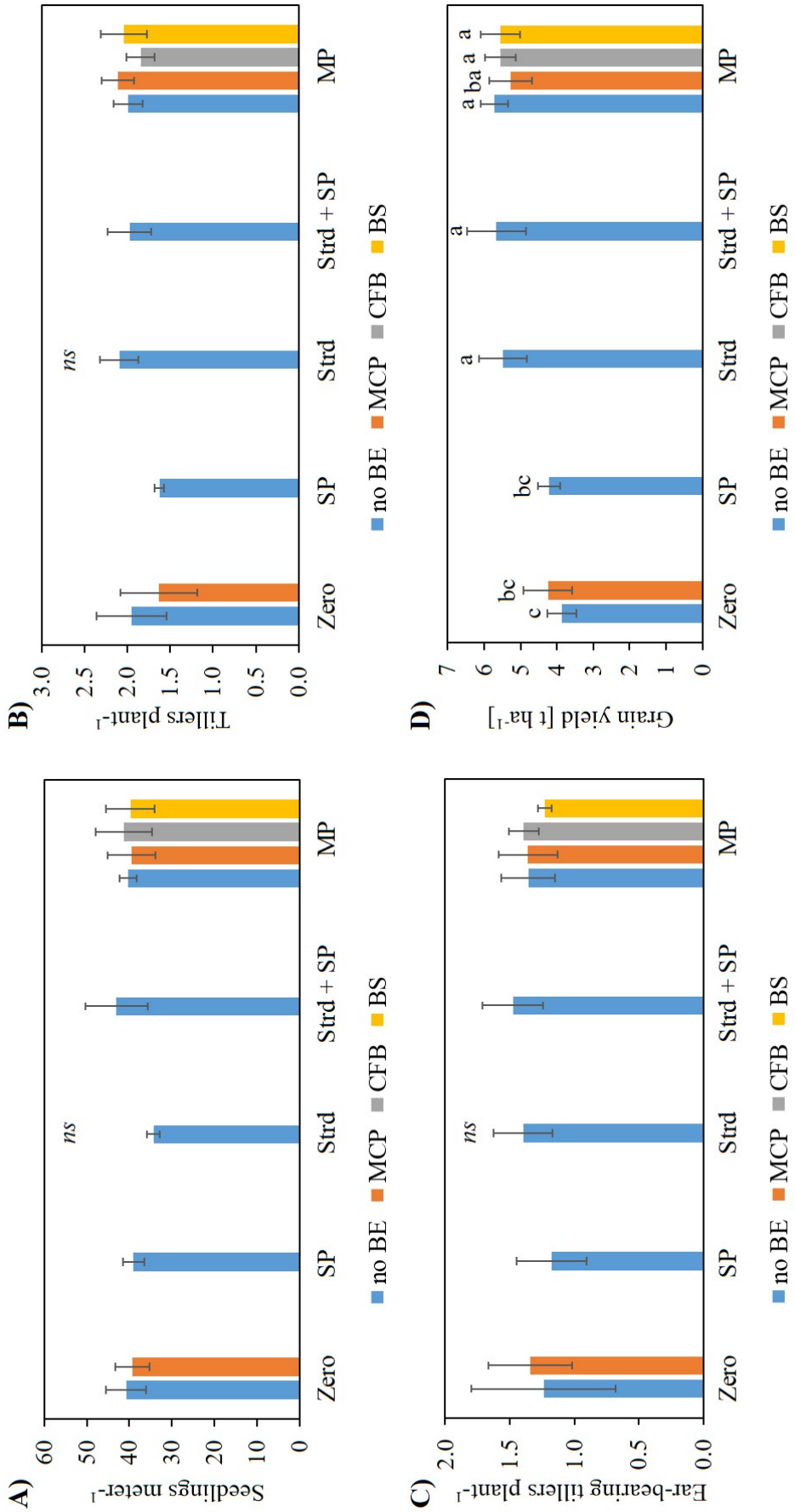

Fig. 7: **A)** Seedling emergence of spring wheat (cv. Alora) at 39 DAS. **B)** Number of tillers per plant at 58 DAS. **C)** Number of ear-bearing tillers per plant at 131 DAS. **D)** Average grain yield at final harvest. Zero = unfertilized; SP = super phosphate; Strd = calcium-ammonium nitrate (CAN); MP = pelleted poultry manure; no BE 0 uninoculkated control, MCP = microbial consortia product; CFB = Combifector-B; BS = *Bacillus subtilis* CH13. All data are presented as mean values with SD (standard deviation) of four replicates Different letters indicate significant differences between treatments (2-way ANOVA, Tukey-Test, $\alpha < 5$ %).

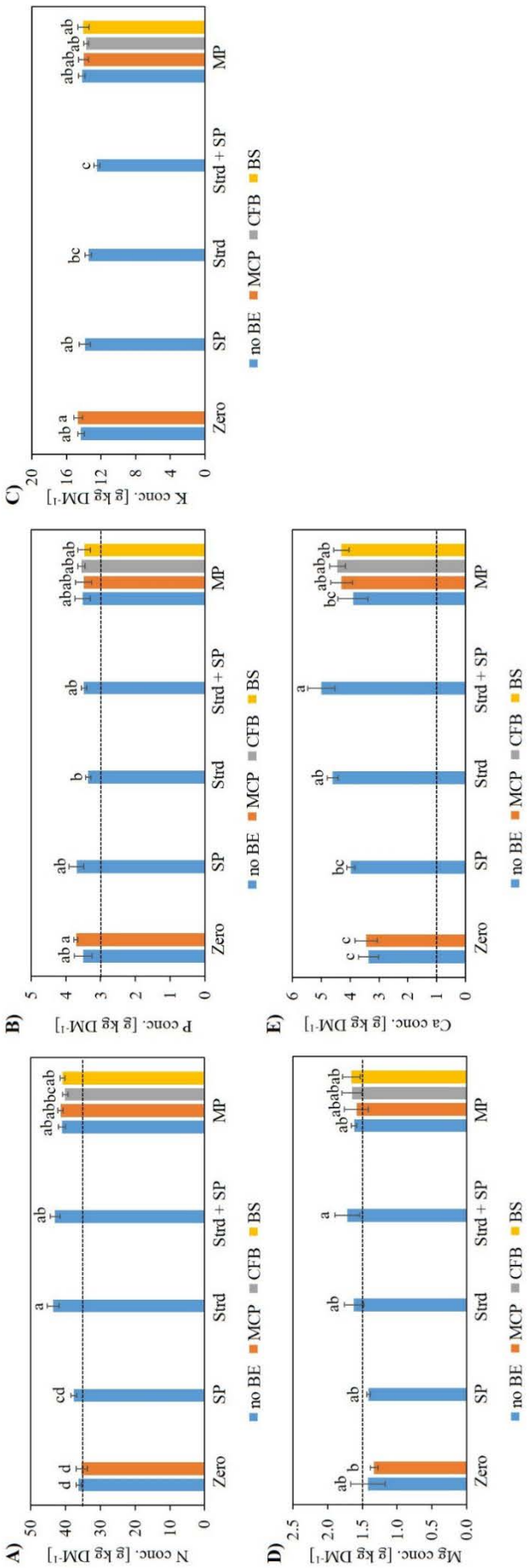

Fig. 8: **A)** Average N concentration in flag leaf (sufficiency range: 35.0 – 43.0 g kg DM^{-1}. **B)** Average P concentration in flag leaf (sufficiency range:3.0 – 4-0 g kg DM^{-1}. **C)** Average K concentration in flag leaf. (sufficiency range: 29 – 37 g kg DM^{-1}) **D)** Average Mg concentration in flag leaf [g kg DM^{-1}]. The optimal Mg concentration in this growth stage is above 1,5 g kg DM^{-1}. **E)** Average Ca concentration in flag leaf [g kg DM^{-1}]. (sufficiency range:>1 g kg DM^{-1}). Nutrient concentrations were measured at 73 DAS. The dotted line indicates nutrient deficiency thresholds (Bergmann, 1988). Zero = unfertilized; SP = super phosphate; Strd = calcium-ammonium nitrate (CAN); MP = pelleted poultry manure; no BE 0 uninoculkated control, MCP = microbial consortia product; CFB = Combifector-B; BS = Bacillus subtilis CH13. All data are presented as mean values with SD (standard deviation) of four replicates. Different letters indicate significant differences between treatments (2-way ANOVA, Tukey-Test, $\alpha < 5$ %).

To test the hypothesis of inadequate rhizosphere establishment of the PGPM inoculants due to drought stress effects and/or pesticide incompatibility as potential causes for the absence BS effects in the field experiment, an additional pot experiment was conducted with controlled water supply, using the same soil and fertilization regime. Plants were cultivated for three weeks with adequate irrigation at 70% water holding capacity (WHC) of the substrate, followed by an 18 d drought stress phase at 40 % WHC and 8 d recovery at 70 % WHC. Contol variants received adequate watering throughout the culture period. BS inoculation was conducted at sowing and at 14 and 28 DAS to achieve optimum conditions for root colonization.

The drought stress treatments significantly reduced shoot biomass production and induced irreversible leaf damage (chlorosis/necrosis), still detectable at the end of the one-week recovery period in the unfertilized control and in the treatments with full mineral or organic (MP) fertilization. Plants with organic MP supply showed lower shoot biomass production but higher drought stress-induced leaf damage as compared with the positive controls with mineral fertilization (**Fig. 9**)

Among the tested BS treatments only the single-strain inoculant *Bacillus subtilis* CH13 in combination with organic MP fertilization had drought protective effects in terms of increased shoot biomass production (+21 %) and reduced drought-induced leaf damage (- 42 %) at the end of the recovery phase (**Fig. 9**).

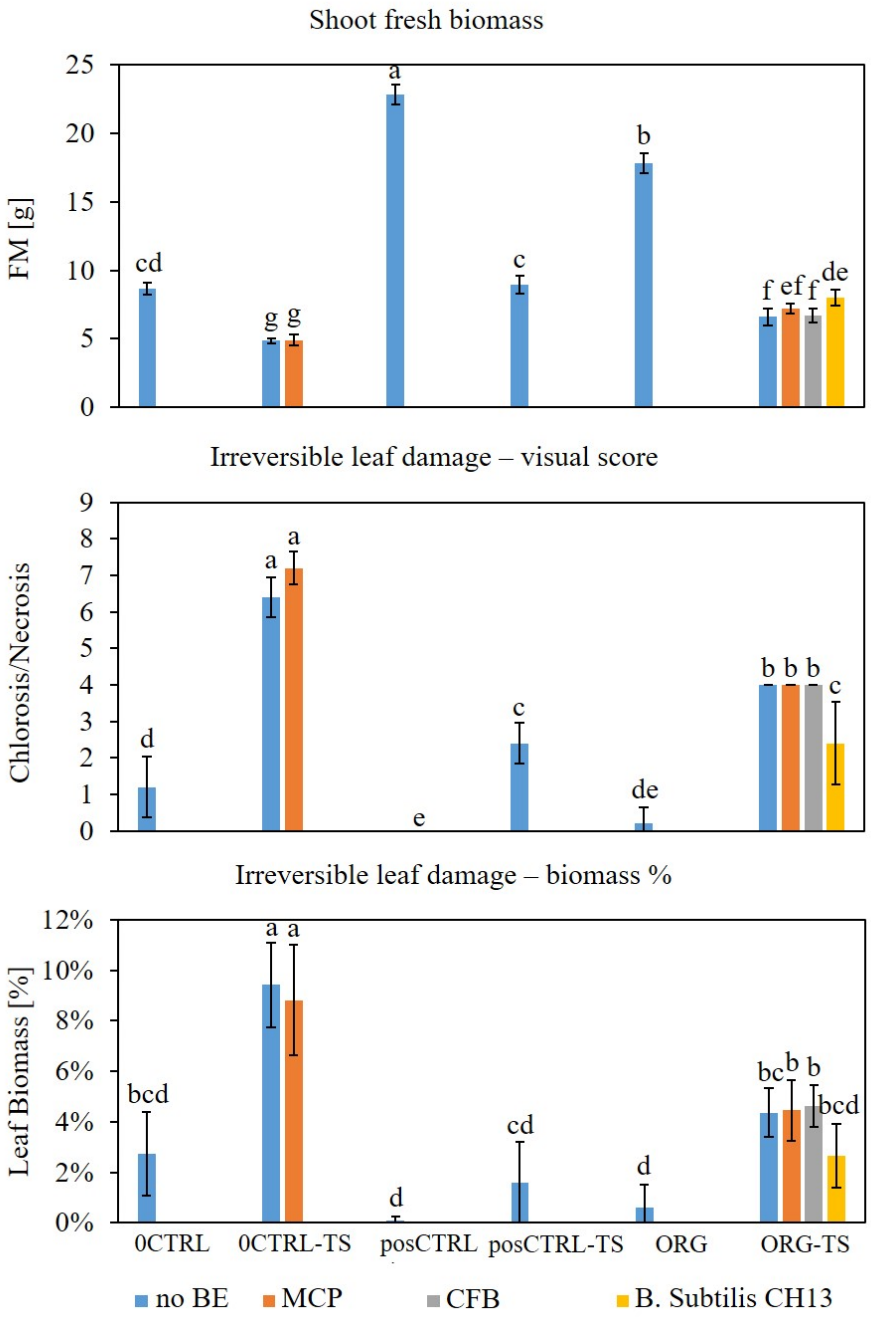

Fig. 9: Shoot biomass and drought stress-induced leaf damage of spring wheat (cv. Alora) in a pot experiment at 43 DAS with and without exposure to a 18 d drought stress period with 40% substrate water-holding capacity (TS); 0 Ctrl = unfertilized; posCTRL =full mineral N, P, K, Mg fertilization; ORG = organic fertilization with pelleted poultry manure; NoBE = uninoculated control; MCP = microbial consortia product; CFB = Combifector B. Means of five replicates. Significant differencres (Tukey Test $\alpha < 5$ %) are indicated by different characters.

This was associated with an improved macronutrient (N, P, K) status of the drought-stressed plants with organic MP fertilization (**Fig. 10**), suggesting that at least the *B. subtilis* CH13 strain was able to improve the utilization of the organic manure fertilizer under drought

stress conditions. However, the plant macronutrient status was critical for all treatments (Bergmann 1988) with the best performance in the positive control with full mineral nutrient fertilization (**Fig. 10**).

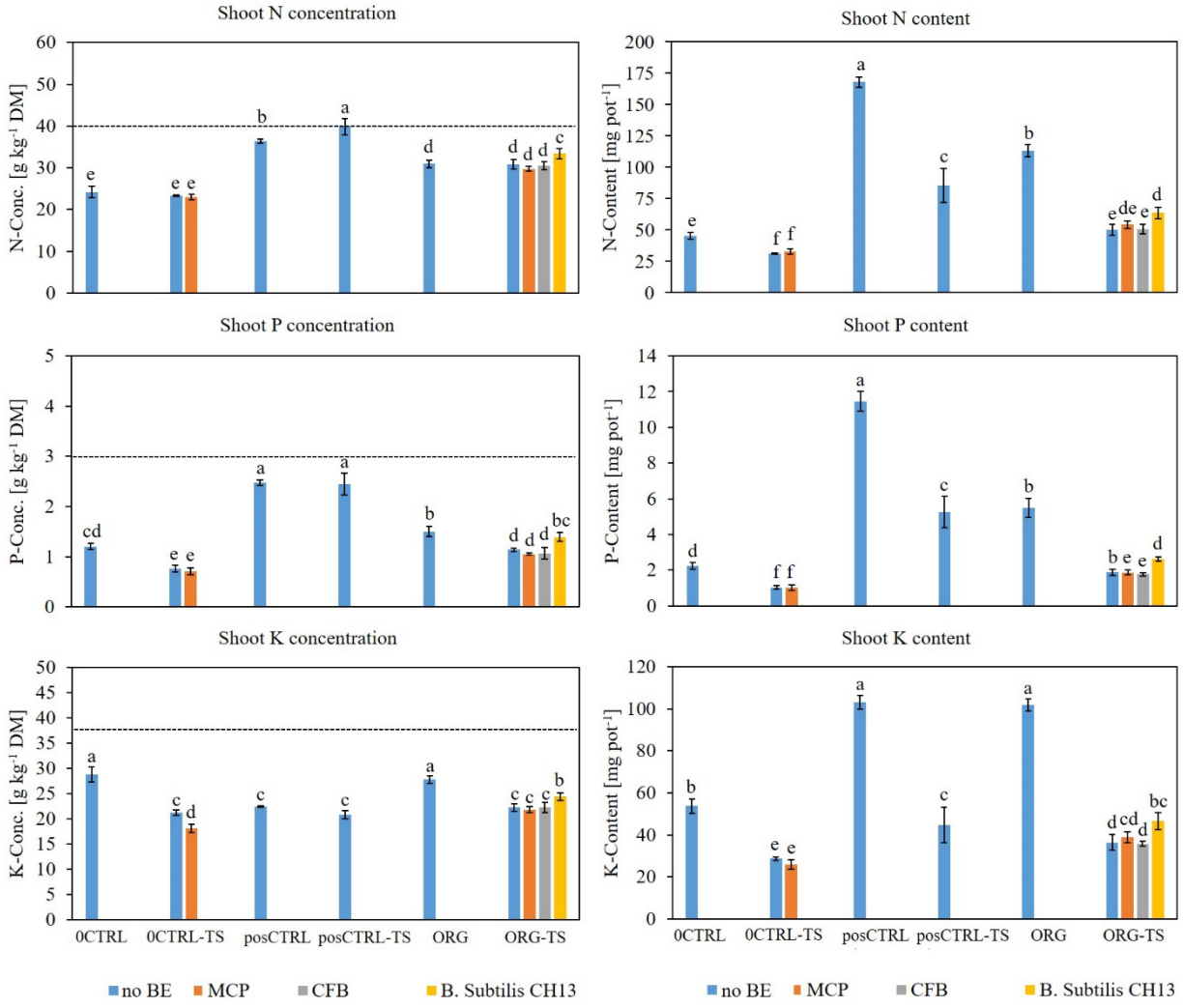

Fig. 10: Shoot concentrations and contents of macronutrients (N, P, K) in spring wheat (cv. Alora) in a pot experiment at 43 DAS with and without exposure to a 18 d drought stress period with 40% substrate water-holding capacity (TS); 0 Ctrl = unfertilized; posCTRL = full mineral N, P, K, Mg fertilization; ORG = organic fertilization with pelleted poultry manure; NoBS = uninoculated control; MCP = microbial consortia product; CFB = Combifector B. Means of five replicates. Significant differencres (Tukey Test $\alpha < 5$ %) are indicated by different characters.

Conclusion

Taken together the experiments could not confirm the hypotheses of improved utilization of organic fertilizers and increased drought resistance of spring wheat induced by the BS inoculants under field conditions. Beneficial effects, observed for BS-induced utilization of the organic MP fertilizer in the pot experiment, avoiding drought stress already durng the establishment phase, underline the importance of protected conditions during the establishment of microbial BS in the rhizosphere. However, contrary to the initial hypothesis of superior MCP performance, significant inoculant effects were recorded only for the single-strain inoculant *Bacillus subtilis* CH13. Similar to the results described by Bradáčová et al. (2019 b, c), the results suggest that the use of MCP inoculants is not always associated with an extra benefit under unfavourable environmental conditions, and the exploitation of beneficial MCP effects requires further investigation with respect to the most suitable application conditions.

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6 General discussion

This research project was initiated to characterize the modes of action and the potential advantages of a representative microbial consortium product (MCP, based on biostimulant (BS) combinations of multiple fungal and bacterial PGPMs and algae extracts), over selected single strain PGPM inoculants and single strain combinations with documented effects on plant growth promotion and pathogen suppression in BS-assisted plant production strategies. Pot and field experiments were conducted with three crops (maize, spring wheat, tomato) on seven different soils with three organic and inorganic fertilization regimes.

6.1 MCP performance as affected by the fertilization regime

Based on the hypothesis that the use of MCP inoculants with multiple microbial and non-microbial BS increases the flexibility in supporting plant nutrient acquisition from various organic and inorganic sources (Lopez-Cervantes and Thorpe, 2013; **Fig. 11**), MCP performance was tested in combination with a range of inorganic (N, P) and organic fertilizers (composted cow manure, poultry manure, meat-, hair-, and feather- meals) in maize, spring wheat and tomato.

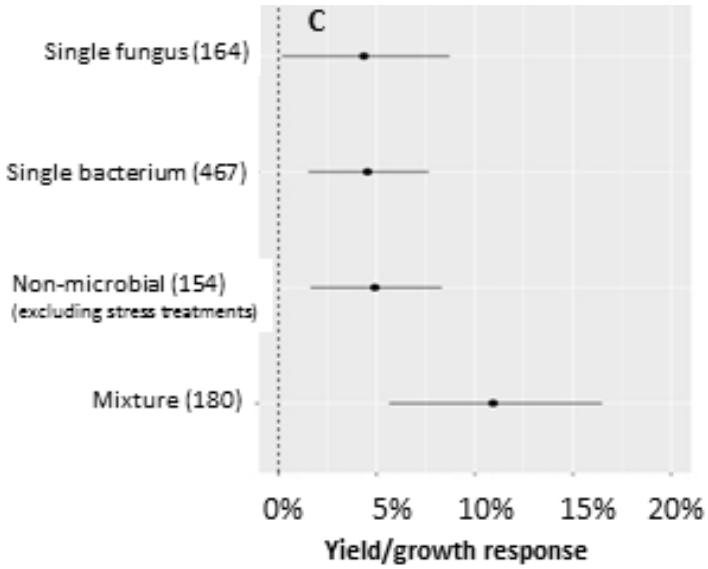

Fig. 11: Yield and plant growth effects of biostimulant (BS) applications, depending on the type and combination of inoculants. The number inside the brackets represents the number of observations included; the dashed vertical zero line indicates no difference between BE and non-inoculated control treatments, the points indicate the mean effect while the horizontal line represents the 95% confidence interval (CI). If CI lines cross the zero line, effects are not significant (BIOFECTOR Final Report; 2017).

6.1.1 MCP interactions with mineral fertilizers

In pot experiments with maize, nitrate fertilization was compared with the application of ammonium fertilizers, frequently used as N starter supply in maize cultivation systems. Benefits of ammonium fertilizers have been attributed to root attracting properties, reduced risk of N-leaching and the potential to induce rhizosphere acidification to increase the availability of P and micronutrients (Nkebiwe et al., 2016a). Nitrification inhibitors were used to ensure a longer-lasting ammonium effect. The experiments were conducted on five soils with moderate to low P availability and a pH range between 5.9 and 7.8 with native soil P, soluble CaH_2PO_4 or sparingly soluble rock-phosphate (rock-P) as P sources.

Generally, beneficial MCP effects on plant growth were most strongly expressed in combination with stabilized ammonium fertilization particularly under conditions of moderately low mineral P availability (20-30 mg kg^{-1} substrate) supplied as soluble fertilizer P or in form of native soil P. This is in line with earlier reports of Mpanga et al. (2019a, b) on performance of various single strain inoculants and single strain combination products and is obviously not an exclusive MCP feature. An overview comprising six experimental variants out of three MCP experiments (Bradáčová et al., 2019a, b), revealed combined effects of ammonium fertilization versus nitrate supply and additional MCP inoculation, mediating improved P acquisition and plant growth (**Tab. 3**). The ammonium effect was obviously related with increased P solubility due to ammonium-induced rhizosphere acidification, which was even detectable on a moderately acidic soil at pH 5.9 (Bradáčová et al., 2019b). By contrast, the additional MCP effect was rather associated with root growth promotion, which was not detectable in the ammonium treatments without MCP inoculation (**Tab. 3**). Accordingly, the increased root length in the MCP variants (**Tab. 3**) mediated improved spatial nutrient acquisition in general and not only P acquisition as observed for the ammonium treatment (Bradáčová et al. 2019b).

Tab. 3: Additive effects of stabilized ammonium supply and MCP inoculation (% increase in comparison with nitrate fertilization) on shoot biomass production and shoot P accumulation and root length development in maize. Average responses calculated from six experimental variants in three pot experiments (Bradáčová et al. 2019a, b).

	NH_4^+ Effect (%)	MCP Effect (%)	NH_4^+ + MCP Effect (%)
Shoot Biomass	13.3 ± 6.2	15.9 ± 8.2	29.3 ± 17.9
Shoot P Content	23.0 ± 9.6	19.2 ± 14.0	47.2 ± 5.0
Root length	3.4 ± 2.2	29.3 ± 8.0	32.6 ± 6.6

The results are very similar to the compilation of 20 experimental variants on ammonium interactions with in total 16 single-strain inoculants, single strain combinations and MCPs reported by Mpanga (2019) for pot and field experiments with maize, wheat and tomato (**Tab. 4**). However, in this case the effects were more intensively expressed as compared with the compilation of MCP trials shown in Table 1. This finding suggests no superior performance of MCP application over the selected single strain inoculants and single strain combinations described by Mpanga (2019) in terms of interactions with stabilized ammonium fertilizers.

Tab. 4: Additive effects of stabilized ammonium supply and PGPM inoculation (% increase in comparison with nitrate fertilization) on shoot biomass production and shoot P accumulation of the host plants. Average calculated from 13-20 experimental variants (pot and field experiments with maize, wheat, tomato and 16 PGPM inoculant strains) on low P soils with sparingly soluble Ca-P sources in comparison with the effects of soluble P fertilization (Mpanga, 2019)

	NH_4^+ Effect (%)	PGPM Effect (%)	NH_4^++PGPM Effect (%)	% of Soluble P Fertilization
Shoot Biomass	35.8 ± 12.5	31.4 ± 6.2	68.7 ± 17.7	84.2 ± 5.8
Shoot P Content	90.6 ± 22.0	11.4 ± 5.1	102 ± 25.4	79.1 ± 6.0

Similar to the results reported by Mpanga et al. (2019 a, b) for various single strain inoculants, ammonium fertilization mediated mainly P solubilization while the MCP inoculants additionally promoted root growth (**Tab. 3**).

Stimulation of root growth induced by various PGPMs is well-documented in the literature (Glick, 2014). The underlying modes of action are based on PGPM-mediated production of phytohormonal signals such as auxins from precursors released by plant roots (Hartmann et al., 2009) or can be stimulated by microbial production of signal compounds interfering with auxin production and hormonal signaling of the host plant, such as certain quorum sensing metabolites or volatile organic compounds (VOCs) (Hartmann et al., 2014; Garnica-Vergara et al., 2015). Another strategy is the enzymatic degradation of the ethylene precursor 1-aminocyclopropane carboxylic acid (ACC) via microbial production of ACC-desaminase, which counteracts excessive stress-induced ethylene production with inhibitory effects on root growth (Glick, 2005). Also improved resistance to biotic and abiotic stress factors induced by microbial signals (e.g. QS metabolites, VOCs) can finally contribute to improved root development (Hartmann et al., 2014; Lee et al., 2016; González-Pérez et al., 2018; Sharifi et al., 2018).

However, the capacity for auxin production by rhizosphere bacteria seems to be dependent also on the form of N supply and was found to be stimulated particularly by ammonium application to artificial growth media in various PGPM strains of the genera Bacillus, Pseudomonas and Acetobacter (Patil, 2011; Bharucha et al., 2013; Mpanga et al., 2019b) Accordingly, Mpanga et al (2019b) found increased auxin production of bacterial populations re-isolated from the rhizosphere of maize plants with stabilized ammonium fertilization after inoculation with *Bacillus velezensis* FZB42 or *Pseudomonas* sp. DSMZ 13134, which was confirmed also for maize plants with MCP inoculation in the present study (Bradáčová et al., 2019b). This points to a mode of action by MCP-induced root growth stimulation via microbial auxin production, which may be further promoted by ammonium-induced auxin accumulation of the host plant (Mpanga et al., 2019b; Moradtalab et al., 2019). A direct effect of microbial auxins on root growth is also indicated by increased expression of the AuxIAA5 gene in the root tissue of MCP-inoculated maize plants, as a member of auxin early response genes, which is known to show rapid upregulation in response external auxin application (Park and Hasenstein, 2015; Bradáčová et al., 2019b). Accordingly, the expression of PIN1c gene, which encodes an auxin efflux transporter involved in shoot-to-root translocation of auxins (Li et al., 2018), known to be rather activated by internal shoot-borne auxin supply, was not activated by MCP application. By contrast, a completely different scenario was observed after inoculation of maize plants with a single strain combination of *Trichoderma harzianum* OMG16 with five strains of *Bacillus licheniformis*, *B. megaterium*, *B. polymyxa*, *B. pumilis* and *B. subtilis*, which induced gene expression of PIN transporters and auxin biosynthesis but not AuxIAA5

expression, responsive to external auxin supply (Moradtalab et al., 2019). Obviously, in this case, instead of microbial auxin production, microbial signals interfered with internal hormonal signaling of the host plant as previously reported for various VOCs produced by Trichoderma inoculants (Garnica-Vergara et al., 2015).

However, the expression of beneficial ammonium-MCP interactions on plant growth was found to be influenced by a range of external factors, mainly related with the efficiency of the ammonium fertilization:

(i) As demonstrated by Bradáčová et al. (2019b), the expression of beneficial MCP effects was strongly dependent on the availability of ammonium, which is largely determined by the stability of the nitrification inhibitors in soils, usually limited to several weeks or months (Benckiser et al., 2013). This implicates that in maize cropping systems, where ammonium is frequently supplied as a starter fertilizer beneficial MCP effects can be mainly expected during early growth, known as a critical phase for proper field establishment of maize (Hajabbasi and Schumacher, 1994; Liu et al., 2016). This was confirmed also in field experiments with single strain inoculants and combination products reported by Mpanga et al. (2019a). The establishment of longer lasting effects might be possible by combination of MCPs with ammonium depot fertilization (see Bradáčová et al. 2019c) or by repeated applications with fertigation systems.

(ii) On light and moderately acidic soils with low pH buffering capacity, beneficial interactions of ammonium fertilization with BS inoculants were occasionally found to be limited by excessive rhizosphere acidification leading to Ca and Mg deficiencies and inhibitory effects on root growth even when P availability was increased (Bradáčová et al., 2019b). This problem was observed for single strain and for MCP inoculants as well (Mpanga 2019; Bradáčová et al. 2019b) but might be overcome by simultaneous application of rock phosphates or other fertilizers increasing the soil pH buffering capacity (e.g. ashes, slags) with protective functions against ammonium-induced over-acidification of the rhizosphere (Mpanga, 2019).

(iii) On the other hand, ammonium-induced rhizosphere acidification is frequently limited on substrates with a high pH buffering capacity, such as alkaline and calcareous soils. Under these conditions, limited performance of ammonium-PGPM combinations has been reported for single strain inoculants (Mpanga et al. 2018; Mpanga 2019) and the investigated MCP product as well (Bradáčová et al., 2019b).

This finding also points to a limited solubilizing potential of the inoculants for acid soluble soil P forms (i.e. calcium phosphates) without additional support via root-induced rhizosphere acidification induced by ammonium supply. Placement of ammonium fertilizers may offer a perspective to overcome this problem, as demonstrated by Jing et al. (2010). Root-attracting properties of the ammonium depot are able to induce the proliferation of short lateral roots close to the depot zone (Liu and v. Wirén, 2017), which leads to an intensification of the rhizosphere acidification effect even in alkaline soils with pH ≈ 8. This effect may be further promoted by root growth-stimulating properties of PGPM inoculants (Nkebiwe et al. 2016; **Fig. 12**).

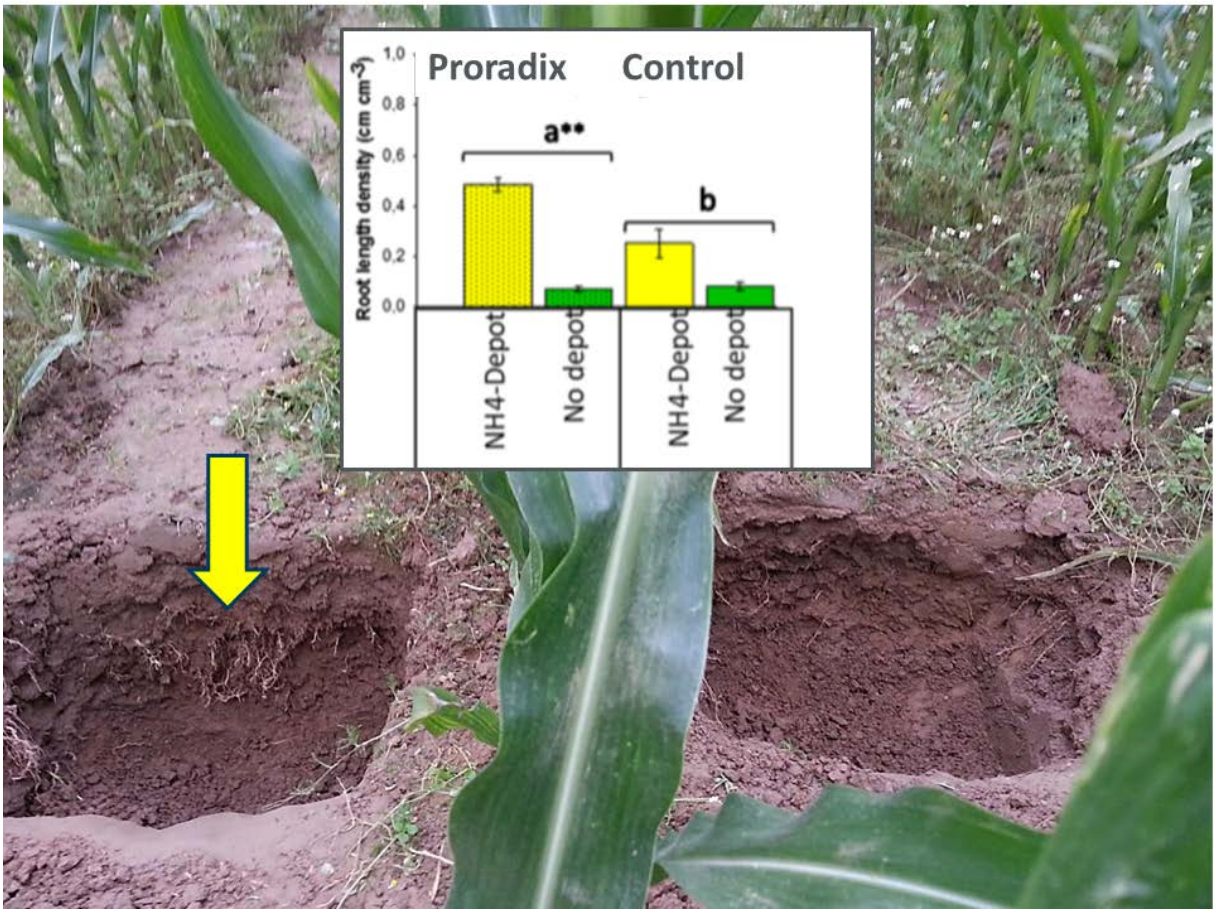

Fig. 12: Localized root proliferation induced by ammonium band placement, stimulated by PGPM inoculation (Proradix = *Pseudomonas sp.* DSMZ 13134) of field-grown maize plants (modified after Nkebiwe et al. 2016).

This approach was tested in an open-field, drip-irrigated tomato production system on a sandy soil with low P availability (P_{Olsen} 5.5 mg kg^{-1}, pH_{CaCl2} 7.9) at the Ramat Research station in the Negev desert in Israel, with band placement of stabilized ammonium

sulfate. Under these conditions, MCP inoculation significantly promoted early growth and final yield of tomato by improved acquisition of native soil P, and could partially replace the effects of soluble P fertilization. The MCP inoculation was superior in comparison with the application of selected single-strain inoculants and strain combinations. This may be attributed to the combined effects of ammonium-induced rhizosphere acidification promoted by fertilizer placement, the ability of tomato to acidify the rhizosphere under P limitation (Pilbeam et al., 1993; Neumann and Römheld, 1999) and additive benefits provided by the various MCP inoculant strains (Bradáčová et al., 2019c).

6.1.2 MCP interactions with organic fertilizers

Improving soil fertility and plant nutrient availability by stimulation of processes involved in C, N and P cycling in the rhizosphere is discussed as a major mode of action of MCP inoculants (Lopez-Cervanets and Thorpe, 1013; Nuti and Giovanetti, 2015; Woo and Pepe, 2018). To test this hypothesis, marker enzymes such as cellulases, glucanases, peptidases and acid and alkaline phosphatases were measured in the rhizosphere of MCP-inoculated and non-inoculated maize plants on soils with different organic matter content (Bradáčová et al., 2019a, b). Beneficial effects on plant growth, induced by the MCP inoculants were recorded on freshly collected field soils, characterized by high rhizosphere marker enzyme activities already in non-inoculated controls. However, MCP-induced plant growth promotion was not associated with a further increase in the activities of the investigated marker enzymes, suggesting that MCP-mediated nutrient mineralization was not a major factor contributing to the beneficial effects of the inoculants, which could be mainly attributed to root growth promotion (see section 7.1). This has been similarly reported for various single-strain inoculants with plant growth promoting potential (Mpanga et al. 2019b; Eltlbany et al., 2019) and may be due to the fact that large fractions of native organic N and P pools in soils are frequently not readily available for mineralization processes due to limited solubility (i.e. phytates) or sequestration in complex polymeric humic substances and the microbial biomass (Schmid-Rohr et al., 2004; Singh and Rengel, 2007; Irshad et al., 2012).

However, a different situation may apply for the application of organic fertilizers with easily available organic and inorganic N and P forms. Accordingly, improved utilization of N-rich organic fertilizers, such as composted manures, guano, meat-, hair-, and feather-meals, has been repeatedly demonstrated in combinations even with the same inoculants investigated above (Thonar et al., 2017; Mpanga et al., 2018; Vinci et al. 2018a, b). Similar responses were

recorded also in open-field organic tomato production trials conducted over three years in Hungary within the framework of the BIOFECTOR project using organic meat- and bone-meal fertilizers, (**Tab. 5**).

Tab. 5: Tomato yields with different single strain inoculants (*Trichoderma harzianum* T22; Pseudomonas sp. DSMZ 13134 (Proradix); Bacillus velezensis FZB42 (Rhizovital) compared with non-inoculated controls (no BE) in field trials conducted over three years in Hungary with organic fertilization based on meat-, and bone-meal (modified after BIOFECTOR Final Report, 2017).

2015		Yield	Yield change		BE Benefit	BE costs
Treatment	Bioeffector	t/ha	t/ha	%	€/ha	€/ha
Control	**no BE**	**61,6**				
V1	Tricoderma harz. (11 kg/ha)	63,0	1,4	2,3	1120	1021
V2	Proradix (1,5 kg/ha)	79,5	17,9	29,1	14336	1500
V3	RhizoVital (3 l/ha)	105,0	43,4	70,5	34720	227

2016		Yield	Yield change		BE Benefit	BE costs
Treatment	Bioeffector	t/ha	t/ha	%	€/ha	€/ha
Control	**no BE**	**82,72**				
V1	Tricoderma harz. (11 kg/ha)	96,8	14,1	17,1	11288	1021
V2	Proradix (1,5 kg/ha)	109,8	27,1	32,8	21688	1500
V3	RhizoVital (3 l/ha)	120,6	37,9	45,8	30304	227

2017		Yield	Yield change		BE Benefit	BE costs
Treatment	Bioeffector	t/ha	t/ha	%	€/ha	€/ha
Control	**no BE**	**103,69**				
V1	Tricoderma harz. (11 kg/ha)	125,4	21,7	21,0	17384	1021
V2	Proradix (1,5 kg/ha)	143,1	39,4	38,0	31491	1500
V3	RhizoVital (3 l/ha)	131,1	27,4	26,4	21896	227

As a part of this thesis, a similar study was initiated in Timisoara, Romania to compare the performance of selected single strain inoculants, and strain combinations of fungal and bacterial origin (*Penicillium* sp., Proradix, Rhizovital) with MCP treatments over two years in tomato greenhouse production trials. Applied fertilizers were based on composted cow manure (nursery stage) and guano, hair-, and feather-meals during the production phase (Bradáčová et al., 2019c). Similar to the experiments conducted in Hungary (Table 3), the BS treatments consistently increased tomato yields compared with the non-inoculated controls over two years. Beneficial effects were detectable already during early growth in the nursery phase, followed by stimulation of flowering and higher yield and improved fruit size distribution even under conditions of increased pathogen pressure (*Fusarium oxysporum*, *Agriotes lineatus*) during the first year. Similar to the earlier reports, the results demonstrated once again the principle effectiveness of the selected inoculants in promoting the utilization of organic fertilizers rich in easily available N and P sources. The cumulative yield increase ranged between 39 and 84%,

but without superior performance of the MCP or strain combinations over the single strain inoculants. Also in a follow-up study with spring wheat on a clay loam soil pH 5.9 with low P availability but high organic matter content, there was no indication for improved utilization of an organic fertilizer based on poultry manure and meat-meal by MCP inoculation both under field conditions and in a pot experiment (Neundorf, 2018). In the latter case, superior performance was recorded even for a single *Bacillus simplex* CH13 strain (**Fig. 9**). However, in these experiments, water limitation was included as an additional stress factor.

6.2 MCP performance under stress conditions

A major challenge for BS-assisted crop production systems is the limited reproducibility of positive results under more challenging environmental conditions in field applications where plants can be exposed to various biotic and abiotic stresses (Menzies et al., 2011). Particularly for microbial inoculants, high rhizosphere competence and efficient root colonization of the host plant is a pre-requisite for the expression of beneficial PGPM effects (Eltlbany et al., 2019). However, under severe abiotic stress conditions, a proper root colonization by PGPMs can be impaired due to stress-induced limitations of root growth and photosynthesis of the host plant and by low stress tolerance of the inoculants as well. Particularly in the latter case, MCPs could offer an effective alternative, due to the presence of a wider range of inoculant strains, which may differ in their tolerance to biotic and abiotic stresses (Woo and Pepe, 2018). In the experiments conducted in the present study, plants and inoculants were intentionally or unintentionally exposed to a range of stress factors including drought (Neundorf, 2018), high temperatures and severe P limitation (Bradáčová et al., 2019c), potential toxicities due to high manure contents of nursery substrates (Nielsen and Thorup-Kristensen, 2001; Bradáčová et al. 2019c) and increased pathogen pressure (Bradáčová et al., 2019c). Interestingly, in all experiments conducted in this thesis, beneficial effects of both, single strain and MCP inoculants were exclusively observed under conditions avoiding excessive stress exposure at least during seedling establishment and early growth.

For example, no MCP effects on the acquisition of sparingly soluble soil P and plant growth promotion were observed in a maize experiment where the plants suffered from severe P deficiency already during the establishment phase of the inoculants in the rhizosphere (Bradáčová et al. 2019b). By contrast MCP inoculation significantly improved the P acquisition potential of well-developed tomato plants exposed to P limitation after a nursery phase with optimal nutrient supply (Bradáčová et al, 2019c). Similarly, a spring wheat field experiment with organic fertilization and severe drought stress during the first four weeks after PGPM

inoculation, revealed no inoculant effects on plant performance and final yield, associated with a 40% yield reduction in comparison with the average yield expectations recorded for the trial location in Thüringen, Germany (Neundorf, 2018; **Fig. 7D**). By contrast, the avoidance of drought stress during the PGPM establishment phase, in a follow-up experiment with the same soil and fertilization regime, resulted in plant growth promotion even under conditions of drought stress in later stages of plant development, at least for the single strain inoculant *Bacillus simplex* CH13 (Neundorf, 2018, **Fig. 9**). Accordingly, different PGPM inoculants failed to induce plant growth promoting effects in maize after plant exposure to drought stress (30% substrate water holding capacity – (WHC)) during the establishment phase of the inoculants in the rhizosphere (Weber, personal communication, **Fig. 13A**).

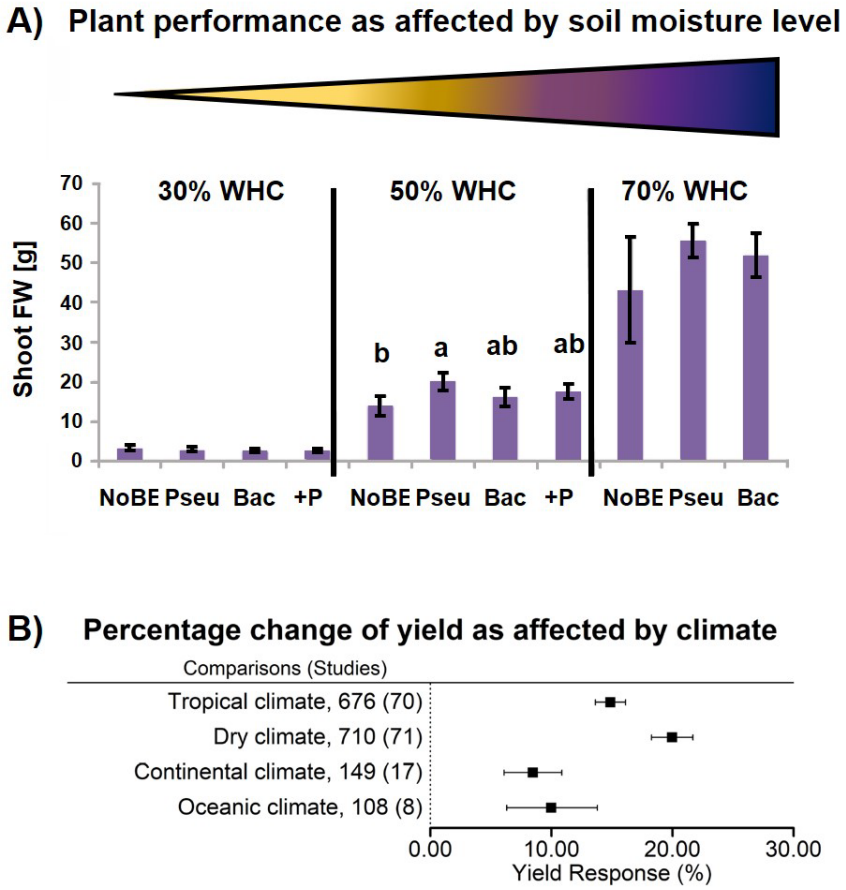

Fig. 13: **A**) Impact of different soil moisture levels (30, 50, and 70 % soil water-holding capacity, WHC) on shoot biomass in a maize pot experiment inoculated with PGPMs (Pseu: *Pseudomonas sp.* DSMZ13134, Bac: *Bacillus velezenzis* 42FZB) or without incoulation (NoBE), with reduced soluble P fertilization (50 mg P kg^{-1} substrate). Full P fertilization (+P) was applied as a positive control at 100 mg P kg^{-1} substrate (Weber, pers. com.) **B**) Meta-analysis covering 166 studies on yield in responses to PGPM application as affected by climate conditions. Mean values and 95% confidence intervals of the back-transformed response ratios are shown. (Schütz et al., 2017).

On the other hand, drought protective effects of PGPM inoculants have been repeatedly reported in the literature as summarized e.g. in the meta-analysis of Schütz et al. (2017) covering 166 publications (**Fig. 13B**). This discrepancy may be explained by the fact that the detrimental effects of drought stress already during the sensitive establishment phase of PGPM inoculants in the rhizosphere is obviously not a widespread scenario. Under these conditions, even the use of more stress-resistant inoculants and MCPs will not provide a significant advantage, as long as the also root growth and root activity of the host plant is severely affected by environmental stress factors. By contrast, a well-established plant-PGPM association is able to express its stress-protective potential. This hypothesis is further supported by comparing the performance of MCP inoculants in horticultural crops, cultivated under protective greenhouse conditions at least during the nursery phase, with MCP responses in an agricultural crop such as maize cultivated under open-field conditions (**Fig. 14**).

Horticultural / vegetable crops: 16 field trials with greenhouse -(pre-)-culture

Crop	No of Trials	Rel. Yield
Tomato	5	27,2 %
Strwberry	4	18,2 %
Cabbage	2	10,3 %
Leak	2	4,9 %
Letuce	3	7,7 %

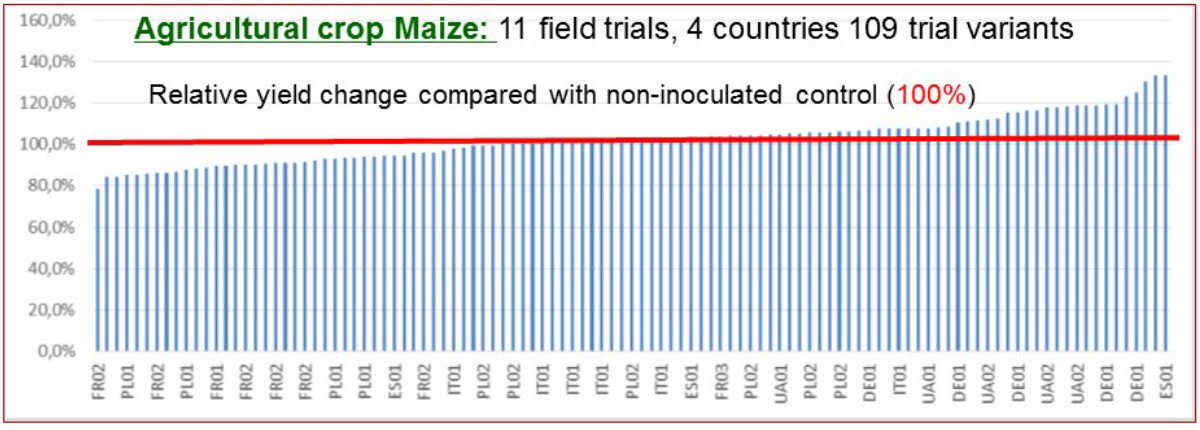

Fig. 14: Yield performance of a microbial consortia product as affected by cropping systems (by courtesy of N. Berger, EurochemAgro GmbH Mannheim, Germany).

6.3 Interactions of MCPs with the soil microbiome

Although clear beneficial effects of MCP inoculants have been demonstrated in this study, it still remains an open question to which extent these effects can be attributed to direct MCP effects on performance of the host plant or to a potential contribution via interactions with the native soil microbiome, since the microbial communities in the rhizosphere are able to influence the physiology and development of plants crucially (Mendes et al., 2013; Berendsen et al., 2012). The soil type and the selective impact of the plant rhizosphere are assumed to have a great impact on the composition of bacterial communities and different microbial communities are harbored in different soil types with specific physio-chemical soil properties (Berg and Smalla, 2009; Lundberg et al., 2012). However, effects of different soil types or the plant developmental stage on bacterial community structures were found to be much more intensively expressed than the often more transient effects of PGPM inoculations (BIOFECTOR Final report, 2017; Eltlbany et al., 2019). This scenario has been demonstrated also for the PGPM effects on tomato performance, investigated in this study (Bradáčová et al., 2019c) and in the framework of the BIOFECTOR project (**Fig. 15**).

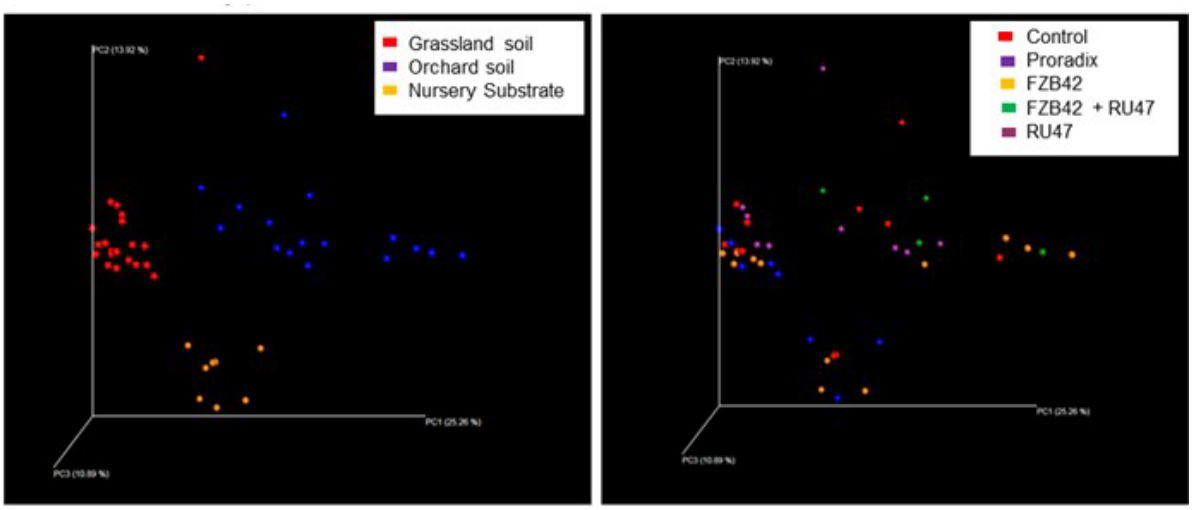

Fig. 15: Three-dimensional PCOA plots of bacterial endosphere communities in tomato plants as affected by the soil type (left panel) and inoculation with different PGPMs (right panel; Control = non-inoculated; Proradix = *Pseudomonas* sp. DSMZ13134; FZB42 = *Bacillus amyloliquefaciens* FZB42; RU47 = *Pseudomonas* sp. RU47) Bacterial endosphere communities in tomato plants are rather affected by substrate properties (left panel) than by PGPM treatments (BIOFECTOR, Periodic Report, 2017).

In all cases described in **Fig. 15**, significant inoculant effects on early growth and yield of the investigated tomato plants were detectable (Bradáčová et al., 2019c; Eltlbany et al., 2019;

BIOFECTOR Final Report, 2017). This points to a strong impact of direct plant-PGPM interactions and not to major changes in rhizosphere bacterial communities as main causes for the observed PGPM effects. The experiments with MCP inoculation conducted in the present thesis further confirm this hypothesis, since beneficial MCP effects e.g. by interactions with stabilized ammonium fertilizers (Bradáčová et al 2019a, b, c) or in combination with different organic fertilizers (Bradáčová et al., 2019c) were similarly recorded on different soils and even with different plant species (maize, tomato) despite the presence of different microbiomes.

However, beneficial effects of microbial inoculants acting via changes in the soil microbiome are also well documented. Examples comprise PGPM-induced promotion of mycorrhizal interactions (mycorrhizal helper effects) documented by Yusran et al. (2009); Thonar et al. (2017) or Eltlbany et al. (2019). This applies also for bio-control effects of microbial inoculants by suppression of soil pathogens (Harman, 2006; Schreiter et al., 2014, Borriss, 2015). Effects on rhizosphere bacterial communities induced by the MCP inoculant were also detectable in this study, reflected in a decline in the abundance of fluorescent Pseudomonades in the rhizosphere of maize, two weeks after the last MCP inoculation (Bradáčová et al., 2019a). In an open field experiment with drip-irrigated tomato, conducted in the Negev desert in Israel, MCP effects on rhizoplane-bacterial communities were recorded even three months after MCP inoculation, although no increased abundance of the bacterial groups harbouring the inoculated strains was detectable (Bradáčová et al., 2019c). The bacterial species richness (alpha diversity) at the rhizoplane declined when the tomato plants were exposed to P limitation but this effect was reverted by MCP inoculation, associated with an improved P nutritional status, growth stimulation and increased fruit yield of the host plants. This may indicate a direct stimulatory effect of the MCP inoculants on the composition of rhizosphere-microbial communities. Alternatively, it may reflect a response to changes in root exudation and rhizosphere pH (Imas et al., 1997; Neumann and Römheld, 1999) triggered by the improved P-nutritional status as a consequence of MCP-inoculation in combination with ammonium fertilization. Moreover, the rhizoplane abundance of *Sphingobacteriia*, known as salinity and drought stress indicators (Lucas et al., 2013; Zhang et al., 2017), declined while the population of potentially plant growth-promoting and drought stress-protective *Flavobacteria* (Kwak et al., 2018) increased in MCP-treated plants. These findings may be interpreted as first indications of some MCP-mediated interactions with the expression of stress-adaptive processes, related with alterations of the rhizosphere microbiome under the challenging climatic conditions at the field site in the Negev desert. However, it remains to be established, if and to

which extent the observed microbiome effects contributed to the plant growth-promoting effects of the MCP inoculant and which changes can be expected in different environments and in combination with different host plants.

In this context, it also remains an open question to which extent the MCP inoculant can contribute to the restoration of beneficial microbial communities in disturbed soil environments? Plant growth-promoting effects in maize were associated with a 60% increased abundance of cultivable rhizosphere bacteria two weeks after MCP inoculation, even on freshly collected field soils. This finding suggests a high initial rhizosphere competence of the inoculants also in presence of highly active, native soil microbiomes, which was indicated by high activities of rhizosphere enzymes involved in C, N and P cycling (Bradáčová et al., 2019a). On a soil with low microbial activity due to long-term (20 years) dry-storage, characterized by low rhizosphere-enzymatic activities, MCP inoculation indicated a moderate increase in enzymatic N and P cycling by 30-40% one week after the last inoculation (Bradáčová et al, 2019b). This may indicate a certain short-term MCP impact on the restoration of microbial nutrient cycling in the maize rhizosphere but this effect was not associated with plant growth promotion. Moreover, the tomato experiment in Israel revealed no effects on the abundance of bacterial taxa present in the MCP inoculant three months after MCP application (Bradáčová et al. 2019c), suggesting no long-lasting rhizosphere survival of the MCP strains, at least under the selected experimental conditions, as similarly reported also for single strain inoculants (Borriss 2015, Bradáčová et al, 2019c). Taken together, the results indicate that MCP application during a single vegetation period revealed no clear evidence for longer-lasting restoration effects of the MCP inoculant. However, the effects of long-term MCP applications over several years remain to be established.

6.4 Concluding remarks and open questions

In the present thesis the plant growth promoting potential of a representative MCP inoculant was investigated in nine experiments (five pot experiments and four field studies) with three crops (maize, wheat, tomato) on seven contrasting soils (pH 5.9- 7.8), ranging from sandy to clay loam. Beneficial effects of MCP inoculation on plant growth and yield formation were detected in five experiments, exclusively under conditions when plant cultivation was performed completely or at least partially under protected greenhouse conditions, particularly during the sensitive rhizosphere establishment phase of the inoculants. In cases without MCP effects, the plants were exposed to stress factors affecting root development such as extreme P

deficiency during early growth, acidic rhizosphere pH and Ca limitation, and drought stress (Neundorf, 2018; Bradáčová et al., 2019b). These findings suggest that MCP rhizosphere establishment, similar to single strain inoculants (BIOFECTOR Final Report, 2017) is affected particularly by stress factors limiting root-development and root activity during rhizosphere establishment. Under these conditions even multiple inoculant strains with differences in stress tolerance will have only a limited advantage, as long as the stress conditions affect the ability of the host plant to support the establishment of a functional MCP interaction in the rhizosphere. Since this scenario is more likely in agricultural crops directly sown under field conditions as compared with greenhouse or nursery cultures, it remains a major challenge for practical applications. Therefore, the selection for stress tolerance or providing stress protected conditions during the establishment phase seem to be of equal importance, both for the microbial inoculants and the host plants as well. This factor is frequently overlooked in the development of PGPM-assisted production systems but is well-documented for special applications such as *Rhizobium* inoculation for improved N acquisition in leguminous plants or the use of arbuscular mycorrhizal fungi as inoculants (Bashan 1998; Kafle et al., 2019). In this context, also further development of application technologies ensuring rapid and efficient root colonization and optimal survival of the inoculants remains an important issue.

In the present study the expression of beneficial MCP effects declined in the order tomato > maize > wheat, as similarly reported in a meta-analysis with the approximately 150 pot and field experiments conducted within the framework of the BIOFECTOR project, comprising 963 experimental variants, investigating the effects of microbial and non-microbial biostimulants (**Fig. 16**). This points to an impact also of genotypic differences in host plant compatibility with the selected biostimulants as an aspect which obviously deserves further attention also in MCP-assisted production strategies.

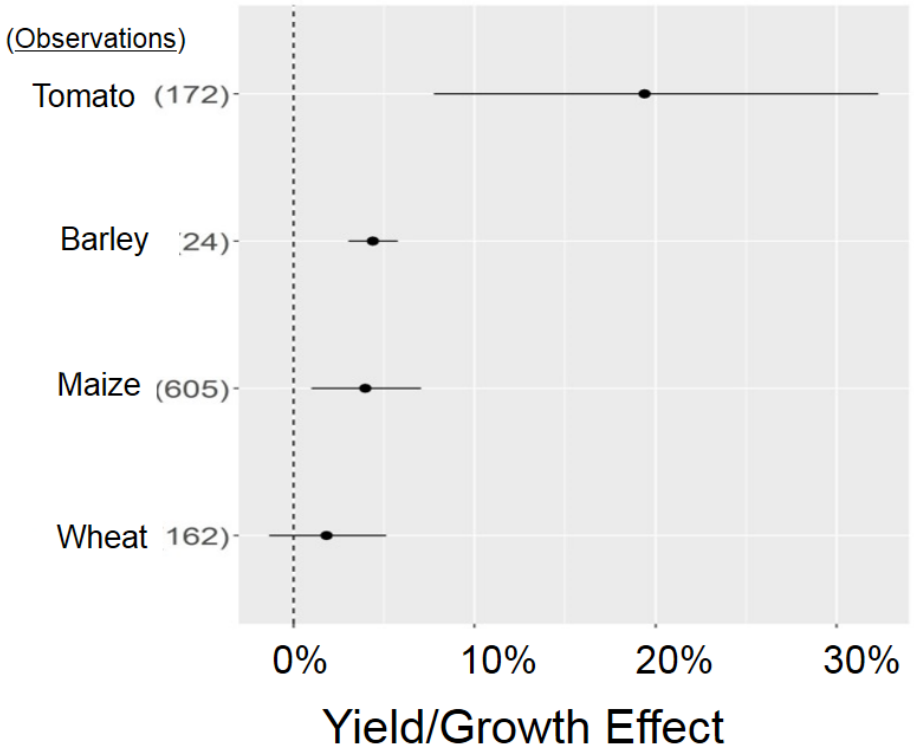

Fig. 16: Yield and plant growth responses to biostimulant applications in different crops. The number inside the brackets represents the number of observations included; the horizontal line represents the 95% confidence interval (CI). If CI lines cross the zero line, effects are not significant (modified after BIOFECTOR Final Report (2017).

The preferential performance of the MCP inoculants in combination with ammonium-dominated fertilization, moderate P availability or N-rich organic fertilizers suggests a selective impact of the fertilization regime on the expression of MCP effects, as similarly reported also for single strain inoculants (Thonar et al., 2017; Vinci et al. 2018a, b; Mpanga et al., 2018; Mpanga et al., 2019a, b). This is an important aspect since it may provide novel management tools to manipulate plant-MCP interactions and to exploit synergistic interactions.

Although, the present thesis demonstrated MCP interactions with the soil microbiome, the significance for plant growth-promoting effects of the MCP inoculants still remains an open question. The same holds true for potential benefits of MCPs over single strain inoculants. Only in one out of nine experiments conducted in this thesis, clear evidence for superior MCP performance was detectable in a drip-irrigated tomato field experiment conducted under the challenging environmental conditions of the Negev desert in Israel (Bradáčová et al., 2019c). This finding demonstrates that MCP inoculants can exhibit an advantage over single strain inoculants but not as a general feature. Therefore, a more detailed characterization of the

conditions promoting superior plant-MCP interaction is required for a more targeted and reproducible MCP application.

Tab. 6 finally summarizes the MCP effects and its main modes of action investigated in the present study under the different production conditions with different crops.

Tab. 6: Overview of all experiments and their major outcomes performed in this study

	Type of experiment					Properties influenced by MCP inoculation								
Nr.		**Crop**	**Fertilization**	**Soil type**	**Soil pH**	**Shoot growth**	**Root growth**	**Yield**	**Nutrient acquisition**	**Microbiome effects**	**Stress mitigation**	**N, C, P cycling**	**Auxin production**
1.	Greenhouse	Maize	NO_3 no P	Silty loam	5.9	-	**0**	**n.d**	**0**	**n.d**	**0 NPdef**	**0**	**0**
	Greenhouse	Maize	NH_4 no P	C_{org} 1.14%	5.9	+	+	**n.d**	+	+	**+ NPdef**	**0**	+
2.	Greenhouse	Maize	NO_3 low P	Clay loam	5.9	**0**	**0**	**n.d**	**0**	**n.d**	**0 Pdef**	**0**	**n.d**
	Greenhouse	Maize	NH_4 low P	C_{org} 2.24%	5.9	+	+	**n.d**	+	**n.d**	**+ Pdef**	**0**	+
3.	Greenhouse	Maize	NO_3 low P	Sandy loam	6.1	**0**	**0**	**n.d**	**0**	**n.d**	**0 Pdef**	+	**n.d**
	Greenhouse	Maize	NH_4 low P	C_{org} 0.58%	6.1	**0**	**(-)**	**n.d**	+	**n.d**	**0 Pdef**	++	**n.d**
4.	Greenhouse	Maize	NO_3 Rock P	Loam	7.6	**0**	**0**	**n.d**	**0**	**n.d**	**0 Pdef**	**n.d**	**n.d**
	Greenhouse	Maize	NH_4 Rock P	C_{org} 0.16%	7.6	**0**	**0**	**n.d**	**0**	**n.d**	**0 Pdef**	**n.d**	**n.d**
5.	Field	Tomato	NH_4 +P depot	Sand	7.9	**0**	**0**	**0**	**0**	+	**0 Pdef**	**n.d**	**n.d**
	Field	Tomato	NH_4 depot no P	C_{org} 0.08%	7.9	+	**0**	+	+	++	**+ Pdef**	**n.d**	**n.d**
6.	Greenhouse	Tomato	MC/FM/Guano	Clay-loam	7.1	+	**n.d**	+	+	**n.d**	**Pathogens?**	**n.d**	**n.d**
7.	Greenhouse	Tomato	MC/HM/FM	Soil/Peat	6.2	+	**n.d**	+	+	**n.d**	**n.d**	**n.d**	**n.d**
8.	Greenhouse	Wheat	Poultry manure	Clay loam	5.9	**0**	**0**	**n.d**	**0**	**n.d**	**0 Drought**	**n.d**	**n.d**
9.	Field	Wheat	Poultry manure	Clay loam	5.9	**0**	**0**	**0**	**0**	**n.d**	**0 Drought**	**n.d**	**n.d**

MC = Manure compost, FM= Feather meal; HM = Hair meal; 0: no effect; +: stimulatory effect; -: inhibitory effect; n.d = not determined
depot = band placement; Pdef = P deficiency; Ndef = N deficiency, NH_4 = stabilized ammonium, NO_3 = calcium nitrate

7 References of introduction and discussion

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